Renal disease progression in autosomal dominant polycystic kidney disease

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Renal disease progression in Autosomal Dominant Polycystic Kidney Disease:

a role for vasopressin?

RIJKSUNIVERSITEIT GRONINGEN

RENAL DISEASE PROGRESSION IN
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A role for vasopressin?

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Chapter 1

Introduction and aims of the thesis

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Introduction

Autosomal Dominant Polycystic Kidney Disease (ADPKD)

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is characterized by progressive cyst formation in the kidneys, leading to renal enlargement (Figure 1), pain, haematuria and end stage renal disease. It is the most common hereditary kidney disease, with a prevalence rate estimated between 1 in 400 (including observed and estimated autopsy cases) and 1 in 1,000 (clinically diagnosed cases only).[1] In the Netherlands, approximately 12,600 people need renal replacement therapy. For more than 1000 people, renal failure is due to ADPKD (8.6%). Of these 1,000 people, 37% undergo dialysis, whereas the other 63% live with a renal transplant (www.renine.nl).

Figure 1: MRI scan of a 45-year old male with ADPKD. Total renal volume is 3091 ml (normal 330 (200-600) ml). Figure derived from[2].

ADPKD is genetically heterogeneous with 2 genes identified. The PKD1 gene is located on chromosome 16, encodes for polycystin-1 and accounts for around 85% of ADPKD cases.[3] The PKD2 gene is located on chromosome 4, encodes for polycystin-2 and accounts for around 15% of cases.[4;5] Whether another gene is involved in a small number of unlinked families is yet uncertain. In general, a mutation in the PKD1 gene is associated with a faster renal function decline than a mutation in the PKD2 gene, but there is considerable overlap.

Current treatment unfortunately is not able to prevent or postpone renal failure. Although successful in numerous renal diseases, there is not much evidence to support dietary protein restriction[6] or strict blood pressure regulation[7] as treatments for patients with ADPKD. The role of the renin-angiotensin-aldosterone system (RAAS) in the pathology of ADPKD is a matter of debate.[8] Renal cyst enlargement is thought to cause bilateral ischemia, and subsequent an increased rennin release. This stimulation of the renin-angiotensin-aldosterone system is supposed to result in both hypertension and progression of cyst growth.[9] However, treatment with angiotensin receptor blockers (ARBs) or ACE inhibitors has yet not been proven effective in prevention of renal function deterioration.[10;11] The lack of finding a beneficial effect could however be explained by the study designs of the trials that have been performed thus far. Most studies had too short follow-up, too small patient groups or included a control group that had almost no renal function deterioration. At the moment, two large scale trials (in early and later stages of ADPKD) are conducted with 4 years of follow-up, in which the impact of intensive blockade of the renin-angiotensin-aldosterone system on progressive renal disease will hopefully

be assessed (the HALT PKD trials).[12;13] Increased understanding of the genetic, molecular and cellular mechanisms underlying ADPKD has resulted in the development of potentially effective treatments.[14] In this introduction, first, current markers of disease severity in ADPKD will be discussed. Second, we will address the role of vasopressin in normal physiology, and its potential unfavorable effects in chronic kidney disease, specifically in ADPKD. Third, we will discuss the potential value of vasopressin receptor antagonists as a new treatment option in ADPKD.

Markers for disease severity in ADPKD

The clinical course of ADPKD is highly variable.[15] In Figure 2, the variability in age at which end stage renal disease is reached, is shown. The figure illustrates that there are differences between ADPKD patients with a mutation in the PKD1 gene and with a mutation in the PKD2 gene. Patients with a mutation in the PKD1 gene have significantly more severe renal disease than patients with a mutation in the PKD2 gene, with larger kidneys[16] and earlier onset at end stage renal disease (median age at death or onset of end stage renal disease is 53.0 vs. 69.1 years).[17] But even within the specific genetic abnormality, there is variation. Furthermore, the figure illustrates that even within families, there is a large spread in age of reaching end stage renal disease.

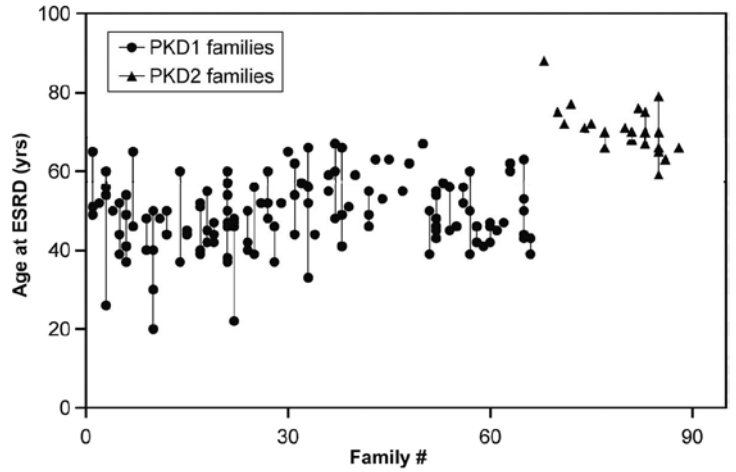

Figure 2: Age plot for ADPKD patients who developed end stage renal disease, grouped by family. Each dot represents the age at which an affected member of the same family developed end stage renal disease. Figure adapted from[18].

With upcoming therapeutic regimens (see further in this introduction) and the existing variable disease course, markers are needed to assess disease severity in ADPKD.

In most renal diseases, kidney function is measured for this purpose. However, measurements of glomerular filtration rate is suggested to be misleading in reporting the progression of ADPKD.[19] Figure 3 illustrates the hypothesis that compensatory hyperfiltration of remaining glomeruli occurs, although glomeruli are lost due to the disease process. Consequently, GFR remains stable during a prolonged period, declining sharply thereafter (grey bullets). Without such a compensatory mechanism, GFR would have declined in a linear fashion (broken line). In black triangles, renal volume increase is depicted, following in parallel with the actual loss of functioning glomeruli. So far however, there is no firm evidence to support this hypothesis.

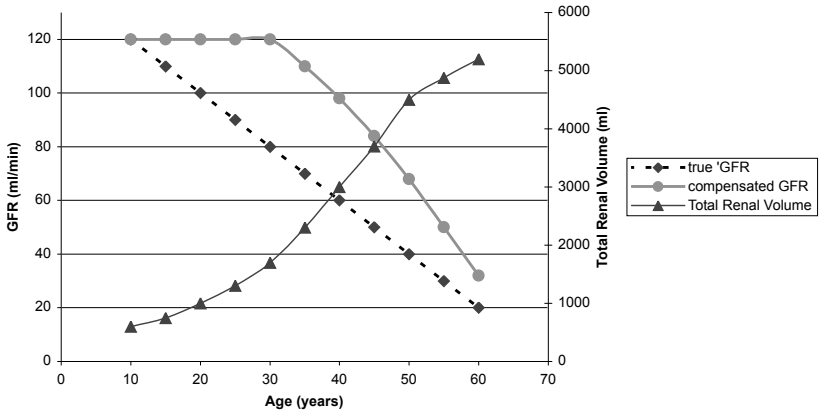

Figure 3: hypothetical associations between age, GFR and total renal volume for a patient with ADPKD. Figure adapted from[19] published in[2].

This hypothesis in mind, it has been suggested that clinical evaluations of potential therapies for ADPKD should better monitor cyst and renal growth to indicate efficacy, instead of preservation of GFR. The Consortium for Renal Imaging Studies in Polycystic Kidney Disease (CRISP) showed that kidney enlargement results from the expansion of cysts in patients with ADPKD. This growth is continuous and quantifiable by MRI and is associated with decline of renal function.[20] Higher rates of kidney enlargement are associated with a more rapid decrease in renal function.[19;20] In a large number of animal studies, treatments that inhibited renal enlargement, also ameliorated renal function.[19] Furthermore, several studies show that the rate of increase of renal volume can reliably be measured.[21-22]
Other proposed markers to define ADPKD severity are renal blood flow and albuminuria. Renal blood flow was measured in a subgroup of 131 patients, drawn form the CRISP cohort, using magnetic resonance imaging. During follow-up, a reduction of renal blood flow paralleled the increase in total kidney volume, and importantly, preceded GFR decline and predicted structural and functional disease progression in this cohort. Several studies showed that in ADPKD, albuminuria is associated with renal volume,[23;24] mean arterial blood pressure, filtration fraction,[24] renal growth and slope of glomerular filtration rate.[25] Albuminuria is regarded to be a valuable biomarker indicating disease severity and predicting outcome. As such it has become a secondary endpoint in intervention studies in ADPKD (Chapter 7).

Vasopressin in normal physiology

The antidiuretic hormone arginine vasopressin is crucial for water regulation in the body (Figure 4). Its secretion is stimulated in response to an increase in plasma osmolarity or a decrease in plasma volume. Vasopressin can bind to three different receptors. The vasopressin 1A receptor is expressed among others on vascular smooth muscle cells and stimulation leads to vasoconstriction.[26] The vasopressin 1B receptor is expressed on brain cells and activation stimulates release of adrenocorticotropin from the anterior pituitary.[27]

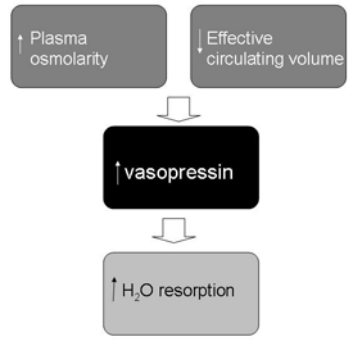

Figure 4: An increased plasma osmolarity and/or a decreased circulating volume stimulates secretion of vasopressin. Vasopressin is derived from a preprohormone precursor that is synthesized in the hypothalamus. It is stored in the posterior pituitary gland to be released into the blood stream in response to the two aforementioned stimuli. Vasopressin mediates water-reabsorption by stimulating the urea-recirculation and the water permeability of the collecting duct.

When vasopressin binds to the vasopressin V2 receptors in the collecting ducts in the kidney, this induces the insertion of the molecular water channel aquaporin-2 in the luminal membrane of principal cells. Activation of the vasopressin V2 receptor also increases the permeability of the inner medullary portion of the collecting duct to urea, allowing increased reabsorption of urea into the medullary interstitium. This increases the concentration gradient created from the removal of water in the connecting tubule, cortical collecting duct, and outer medullary collecting duct. Both processes mediate water reabsorption, thereby reducing water excretion.[28;29] Thus, for concentrating urine, vasopressin is vital. Patients with central diabetes insipidus (who lack vasopressin) or with nephrogenic diabetes insipidus (where there is resistance against the action of vasopressin) experience a marked, sustained increase in water excretion. This requires virtually continuous drinking, day and night, to prevent dehydration, illustrating the importance of vasopressin for normal physiology.

Copeptin as a surrogate for vasopressin

Reliable measurement of vasopressin concentration in blood is problematic. This is caused by the fact that more than 90% of vasopressin in the circulation is bound to platelets. Vasopressin is furthermore, unstable in isolated plasma.[30] Because of the small size of vasopressin, vasopressin cannot be measured by sandwich immunoassay, but only by less sensitive competitive immunoassays.

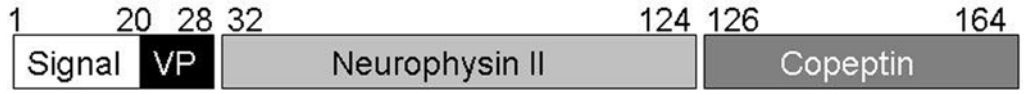

Figure 5: Schematic representation of the peptide precursor of arginine vasopressin, showing the signal sequence (white), vasopressin (VP, black), neurophysin II (light grey) and copeptin (dark grey). Numbers indicate amino acids of the human protein. Figure derived from[31].

Recently, an assay has been developed to measure copeptin, the C-terminal portion of the precursor of vasopressin. Because this copeptin assay is a sandwich immunoassay, it is remarkably sensitive, the detection limit is 1.7 pmol/L. Copeptin has been shown to be a reliable marker of vasopressin secretion and a useful substitute for circulating vasopressin concentration in clinical routine.[31-33] These characteristics make copeptin a promising marker, which allows for the first time (indirect) measurement of vasopressin in large populations. The structure of copeptin is depicted in Figure 5. In Figure 6, the principle of the copeptin sandwich immuno-assay is depicted.

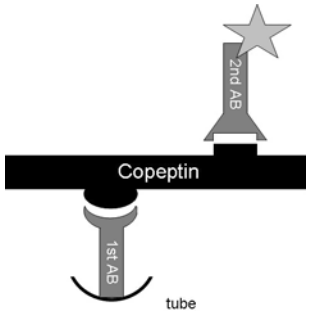

Figure 6: Principle of the copeptin sandwich immunoassay. A polyclonal antibody (1st AB) directed to amino acids 132-147 of human copeptin is bound on the solid phase; a second polyclonal antibody (2nd AB) directed to amino acids 149-164 of copeptin is labeled (star) for chemiluminescence detection. Figure adapted from[31].

Unfavorable renal effects of Vasopressin

Despite its relevance for normal physiology, several unfavorable effects of vasopressin have been reported on the kidney. Experimental studies have shown that a sustained vasopressin V2 receptor stimulation results in increased renal plasma flow and particularly in increased GFR.[34] This hyperfiltration may have deleterious consequences, in particular in diseased kidneys, resulting in renal hypertrophy,[35] proteinuria,[36] and accelerated renal function decline.[37-39] This proposed mechanism is supported by studies in rats with chronic renal failure, showing that increased water intake or chronic infusion of a vasopressin V2-receptor antagonist reduced proteinuria and prevented glomerulosclerosis[37;40] and tubulo-interstitial fibrosis.[41] Similarly, chronic infusion of a vasopressin V2-receptor antagonist reduced albuminuria and kidney weight in diabetic rats.[42] In humans, administration of 1-desamino-8-D-arginine vasopressin (a vasopressin receptor agonist) induced albuminuria.[36]

Unfavorable effects of vasopressin in the pathogenesis of ADPKD

Apart from these general unfavorable renal effects, vasopressin probably also has a specific detrimental role in the pathogenesis of ADPKD. In 1999, it was discovered in the roundworm Caenorhabditis elegans that homologues of polycystin-1 and polycystin-2, the gene products of respectively PKD1 and -2, function as mechanosensors in specialized ciliated sensory neurons. This discovery prompted interest in the role of cilia in mammalian kidneys, specifically in ADPKD. Indeed, also in humans, the polycystins are located in the primary cilium. Other support for the involvement of primary cilia in ADPKD came from studies in rodent models for polycystic kidney disease, which all appeared to have defects in their cilia function.[43;44] Polycystin-1 and polycystin-2 probably form a functional complex,[45] in which polycystin-2 is a non-selective cation channel, capable of transporting calcium ions.[46] In tubular epithelial cells, the

cilium projects into the lumen. It is likely that the polycystin complex acts as a mechanosensor on cilia, detecting changes in tubular urine flow, reacting by calcium influx, possibly through the polycystin-2 channel. This calcium influx induces release of calcium from intracellular stores.1 In turn, calcium controls cell proliferation and regulation through growth factors and hormonal stimulation. Two aspects seem important in cyst formation: proliferation of tubular cells and cyst fluid secretion by these cells. Important players in this proliferation cascade, partly controlled by calcium influx, are adenosine 3',5'-cyclic monophosphate (cAMP)[47] and mammalian target of rapamycin (mTOR).[48]

Activation of the vasopressin V2 receptor on the basolateral side of renal tubular cells will result in an increase in cAMP, which in turn stimulates cell proliferation and fluid secretion and causes cyst enlarging.[49] Stimulation of the vasopressin V2 receptor is caused by vasopressin in the circulation. It is described that ADPKD patients have high levels of vasopressin, compared to healthy controls,[50;51] although the evidence for this is scarce. In addition to that, ADPKD patients also have an impaired urinary concentrating capacity. This might be caused by insensitivity for vasopressin and / or by anatomic disruption of the vasculotubular architecture.[52-55] Apart from the general unfavorable effects of vasopressin, vasopressin will result in an increased intracellular cAMP concentration in ADPKD patients, thus aggravating the cystic disease. How the abnormalities in ciliary function that occur when PKD1 or PKD2 are mutated, are linked exactly to other components of cyst formation (such as cAMP accumulation, chloride-dependent cyst fluid secretion and regulation of mTOR) is not yet completely understood. Figure 7 is a simplified version of the hypothetical pathways in healthy subjects (A) and in patients with ADPKD (B).

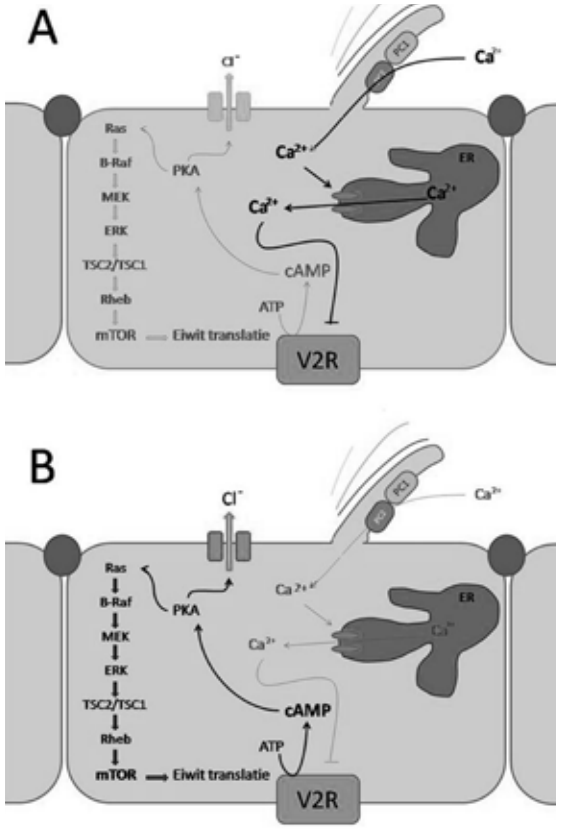

Figure 7: Hypothetical pathogenetic pathways in ADPKD.
Pathways depicted in bold are upregulated, pathways depicted in grey are downregulated.

A. Depicts an epithelial cell of the kidney's collecting duct of a healthy individual. As long as there is urine flow in the tubule, the cilium will bend, inducing a calcium influx through the polycystin complex. This in turn induces release of calcium from the endoplasmatic reticulum. Cyclic AMP is inhibited by this calcium.

B. Depicts this same cell, but now derived from an individual with ADPKD. Because of a defect in the polycystin complex, there is less calciuminflux, resulting in an increased intracellular cyclic AMP concentration. This will induce on the one hand more protein translation, causing a disturbed proliferation, adhesion, migration, differentiation and maturation of the tubulule cells and on the other hand more chloride dependent fluidproduction, enabling the cysts to grow.
How these processes are interrelated is at this moment still unknown. Vasopressin V2 receptor antagonists block the vasopressin V2 receptor (V2R) and thereby inhibit cAMP, inhibitors of mTOR inhibit protein translation. Figure adapted from[1] published in.

Vasopressin V2 receptor antagonists in ADPKD
In the past decade, studies in animal models of cystic renal diseases have suggested a final common pathway for cystogenesis, involving a major role for cAMP-stimulated signaling pathways in controlling both the rate of epithelial cell growth and fluid secretion in cysts.[47;56] Since agonists of cAMP stimulate cell proliferation and fluid secretion and cause cyst enlarging, it follows that antagonists of cAMP-mediated processes could be therapeutic targets to reduce cyst volume. This appeared true for the vasopressin V2 receptor antagonist OPC-31260 (produced by the pharmaceutical company Otsuka) in animal models for autosomal recessive polycystic kidney disease (ARPKD) and nephronophthisis,[57] and one year later in a model for ADPKD. Other experiments support the hypothesis that vasopressin plays an important role in the pathogenesis of ADPKD. By crossing PCK rats (a model for ARPKD, Pkhd1-/-) with Brattleboro rats (AVP -/-), rats were generated with ARPKD and varying amounts of endogenous vasopressin. Administration of a vasopressin V2 receptor agonist recovered the cystic phenotype in this rat model (i.e. it brought back the cysts) without vasopressin and aggravated the cystic phenotype of those rats with ARPKD and with vasopressin but did not induce cystic formation in wild-type rats.[58]

In 2005, the effectiveness of another vasopressin V2 receptor antagonist, OPC-41061 (tolvaptan®, also from Otsuka) was proven in a rat model for ARPKD.[59] This is an interesting finding since OPC-41061 has more affinity for the human vasopressin V2 receptor than OPC-31260. There is already some experience with Tolvaptan as a treatment in humans. In 2009, tolvaptan is approved by the FDA for treatment of hypervolemic and euvolemic hyponatraemia, including patients with heart failure, cirrhosis, and the syndrome of inappropriate anti-diuretic hormone. The EMA approved the drug for treatment of hyponatraemia due to the syndrome of inappropriate antidiuretic hormone. These approvals were based on studies of efficacy and safety in patients with hyponatriaemia[60] and in patients with congestive heart failure, with or without hyponatraemia.[61;62] These studies showed that the drug seems safe and well tolerated, with no major safety issues. An investigational new drug application has been filed by the manufacturer of Tolvaptan to examine its effect in clinical trials for cyst growth modification in ADPKD. Phase I and II trials have been completed. As yet however, no data are available on efficacy of vasopressin receptor antagonists to delay disease progression in ADPKD.

Besides knowledge whether these new drugs will indeed decrease cyst formation in subjects with ADPKD and preserve renal function, we need to learn about the adverse effects of these new drugs, the more since therapy probably has to be given life-long for this chronic condition. What might be problematic of the treatment with tolvaptan is the severe water diuresis and thirst that is induced by this agent. When given to healthy, normally hydrated male volunteers, tolvaptan causes an increase in urine volume (without natriuresis), which is dose dependent over 6 hours and which is increased by up to 4 times compared to placebo.[63] Polyuria and especially nycturia are therefore expected to occur in ADPKD patients and will probably have a negative influence on quality of life. Given this side effect profile, it would be an option to use lower dosages of vasopressin receptor antagonists. This is likely to result in less adverse effects, but also in less effectivity. To overcome this decreased efficacy, a combination therapy

could be instituted with other agents that are now being tested for efficacy in ADPKD. Of note, these agents also have side effects that will limit the probability of lifetime use in high dosages. Examples of such potential agents are mTOR inhibitors, like rapamycin and sirolimus[64] and somatostatin analogues.[65;66] Recent clinical trials did not show a clear beneficial effect of mTOR inhibitors in ADPKD,[67;68] making them less favorable candidates for combination therapy. Finally, when proven effective, an essential question will be when treatment should be initiated: early or later in the disease? Because ADPKD is a progressive condition and probably the cysts that are formed early in life are the main contributor to eventual total cyst volume,[69] it would make sense to start an intervention as early in this process as possible to delay or prevent long-term consequences as renal failure. Furthermore, vasopressin V2 receptor antagonists are believed to be preventive, not restorative. The effect of treatment in a later phase of the disease could therefore be less than treatment in an early phase. On the other hand, an early intervention would mean that every patient with ADPKD will be exposed for a lifetime to the adverse effects of the agents needed to intervene, while not all patients will reach end stage renal disease.[70] Because of these reasons, it will be important to discover markers that identify ADPKD patients with rapid disease progression. In such patients, therapy could be instituted.

Aims of this Thesis

In this thesis, measures of renal damage in ADPKD as well as detrimental effects of vasopressin in renal disease, and specifically in ADPKD, are studied.

Part I focuses on measures of renal damage in ADPKD. With the variable disease course and upcoming potentially effective therapeutic regimens, measurement of disease severity will become more important. Chapter 2 therefore describes renal abnormalities that occur relatively early in the disease. This is of particular interest as assessment GFR is of limited value. Subsequently, in chapter 3 the potential value of urinary biomarkers to assess disease severity is investigated. The advantage of measuring urinary biomarkers compared to renal hemodynamic parameters is that urinary biomarkers are relatively easy to measure and low in costs.

In part II, the focus is on potential detrimental effects of vasopressin. As described above, vasopressin is difficult to measure. Therefore, copeptin is used as a surrogate for vasopressin. In chapter 4, we investigate whether cross-sectionally, copeptin is associated with microalbuminuria in a large general population-based cohort (the PREVEND-study). In chapter 5, we investigate in a cohort of renal transplant recipients the predictive value of baseline copeptin concentration for graft failure and rate of renal function decline during follow-up. Next, in chapter 6, copeptin is investigated in ADPKD. We investigate the potential association between copeptin and disease severity (as assessed by effective renal blood flow, total renal volume and albuminuria).

Part III is the synthesis of the two previous parts: does inhibition of vasopressin ameliorate progression of disease severity in ADPKD? So far, no human data are available on this subject. The first, large worldwide phase III trial is conducted at this moment (the TEMPO study). In chapter 7, the design and baseline characteristics of this study are given. It is a multicenter, double-blind, placebo controlled trial that will for the first time investigate the therapeutic effect, safety and tolerability of a vasopressin V2 receptor antagonist in patients with ADPKD. If proven effective, it will be essential to assess the dose-effect and dose side effect relationships of these drugs to assess at which dose these drugs are effective, while having an acceptable side effect profile. Furthermore, it is important to obtain information whether these drugs are effective in early as well as late stage disease. We therefore investigated the renal effects of different dosages of a vasopressin V2 receptor antagonist at different stages of the disease in an animal model for ADPKD. The results of this experiment are described in chapter 8.
In chapter 9, the results of the previous chapters are described in their context. The implications of these studies and future perspectives are discussed.

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Part I
Chapter 2

Early renal abnormalities in Autosomal Dominant Polycystic Kidney Disease

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Abstract

Potential therapeutic interventions are being developed for Autosomal Dominant Polycystic Kidney Disease (ADPKD). A pivotal question will be when to initiate such treatment, and monitoring disease progression will thus become more important. Therefore, the prevalence of renal abnormalities in ADPKD at different ages was evaluated.

Included were 103 prevalent ADPKD patients (Ravine criteria). Measured were mean arterial pressure (MAP), total renal volume (TRV, MRI), GFR, effective renal plasma flow (ERPF), renal vascular resistance (RVR), and filtration fraction (FF). Twenty-four-hour urine was collected. ADPKD patients were compared with age- and gender matched healthy controls.

Patients and controls were subdivided into quartiles of age (median ages 28, 37, 42 and 52 years). Patients in the first quartile of age had almost the same GFR when compared to controls, but already a markedly decreased ERPF, and an increased FF (GFR 117 ±32 vs. 129±17 ml/min, ERPF 374±119 vs. 527±83 ml/min and FF 32±4 vs. 25±2% and RVR 12(10 to 16) vs. 8(7 to 8) dynes/cm^2, respectively). Young adult ADPKD patients also had higher 24-hour urinary volumes, lower 24-hour urinary osmolarity and higher urinary albumin excretion (UAE) than healthy controls, although TRV in these young adult patients was modestly enlarged (median 1.0L).

Already at young adult age, ADPKD patients have marked renal abnormalities, including a decreased ERPF and increased FF and UAE, despite modestly enlarged TRV and near-normal GFR. ERPF, FF and UAE may thus be better markers for disease severity than GFR.

Introduction

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is the most prevalent inherited renal disease with an estimated prevalence between 1:400 and 1:1000.[1] The disease is characterized by pain, haematuria and most importantly by progressive cyst formation in both kidneys, often leading to end stage renal disease. Annually, 7.8 male and 6.0 female individuals per million of the population start renal replacement therapy in Europe because of polycystic kidneys, which is 6% of the new end-stage renal disease patients.[2]

Current treatment cannot prevent renal failure.[3;4] However, a better understanding of the pathophysiology of the disease and the availability of animal models identified promising candidate drugs for renal preservation.[5] Clinical trials have been initiated for vasopressin-V2 receptor antagonists, long-acting somatostatin analogues and mammalian target of rapamycin inhibitors.[6] When efficacy of these agents has been established, pivotal question will become when to initiate such treatment. Given that ADPKD is a progressive condition, it seems most appropriate to initiate intervention as early in life as possible to delay or prevent long-term consequences, including renal failure and cardiac complications. On the other hand, ESRD occurs in approximately 50% of affected subjects,[7;8] and it is not appropriate to expose those subjects that will not reach ESRD to excessive medical treatment to such an extent as to cause adverse events, especially because all candidate drugs have considerable side effects. Because of these reasons, it will be important to discover markers that identify ADPKD patients who will develop rapid disease progression. In such patients, therapy could be instituted in an early phase.

It will therefore become important to define disease severity in ADPKD. Criteria to make this distinction are not crystal clear. Glomerular filtration rate (GFR) is believed to be stable for a long period, despite progression of renal anatomical abnormalities, because of compensatory hyperfiltration. GFR is therefore assumed not to be representative of disease severity.[9;10] Total renal volume has been proposed as a surrogate marker for disease progression.10 However, despite a significant overall association, there are subjects with a high total renal volume but normal renal function.[11] Another parameter that is decreased early in the disease is urine concentrating capacity.[12] Other candidate markers to define disease severity are albuminuria[13;14] and renal blood flow. [15;16] Despite evidence for the importance of finding early renal abnormalities in ADPKD, systematic evaluation of hemodynamic parameters, especially with respect to renal blood flow, renal vascular resistance and filtration fraction, has received little attention. Therefore, we investigated renal parameters in ADPKD at different ages in comparison to healthy subjects.

Materials and Methods

Patients

One hundred and eighteen consecutive patients with ADPKD visiting our out-patient clinic, meeting our in- and exclusion criteria were asked to participate. Diagnosis of ADPKD was made upon Ravine criteria.[17] Subjects were ineligible to participate if they were on renal replacement therapy, had undergone renal surgery, were unable to undergo magnetic resonance imaging (as having distorting foreign bodies or aneurysmal clips), had other systemic diseases potentially affecting renal function (as diabetes mellitus and malignancies), or had other medical conditions

that included pregnancy, lactation, or who were less than 6 months postpartum. After screening, subjects underwent an extensive medical history. Thirteen patients refused to participate and two patients were not eligible to participate, leaving 103 patients for analyses. Subjects were scheduled for a 1-day outpatient clinic evaluation.
Values of these patients were compared to healthy controls. These were drawn from a pool of subjects who were screened for live kidney donation and underwent the same evaluation, but without MRI (n=103). Values for healthy controls depicted in tables or figures are derived from these live kidney donors. Twenty-four hour urine collection was not available for all donor screenees. Values considering 24h urinary volume, -osmolarity and-albumin excretion were therefore drawn from subjects participating in the Prevention of Renal and Vascular End stage Disease (PREVEND) study (n=103).[18;19] Controls were matched for age and gender with ADPKD patients and were considered healthy in case they had a history without cardiovascular and / or renal disease, used no medication (other than antihypertensive medication, but no ACEi/ ARB). All patients and controls were of Caucasian ethnicity. There was no difference in age (p=0.7), gender (p=0.9), mean arterial pressure (MAP, p=0.4), body surface area (BSA, p=0.5) or body mass index (BMI, p=0.7) between the two control groups (live kidney donors and the subjects from the PREVEND study). Study site and laboratory methods were the same for patients and the control groups (all subjects were seen in one institution).
This study was performed in adherence to the declaration of Helsinki. All subjects gave written informed consent.

Measurements and definitions

Blood pressure was assessed with an automatic device (Dinamap) for 15 minutes during the renal function measurement. Systolic and diastolic blood pressure values were used to calculate mean arterial pressure (MAP) using the standard formula [MAP = 2/3 * diastolic blood pressure + 1/3 * systolic blood pressure]. Patients collected a 24h urine sample prior to the outpatient visit. Weight and height were determined. Body mass index (BMI) was calculated as weight in kilograms (kg) divided by height2 (measured to the nearest 0.5 kg and 0.5 cm respectively). Urinary albumin concentration was determined by nephelometry (BNII; Dade Behring Diagnostics, Marburg, Germany). Blood samples were drawn prior to renal function measurement for determination of serum electrolyte, haemoglobin, creatinine and urea. Concentrations of haemoglobin, sodium, potassium, cholesterol, and glucose were measured in serum or urine using standard methods. Creatinine was measured with the Roche enzymatic creatinine assay. Creatinine values were used to calculate an estimated glomerular filtration rate (eGFR), using the abbreviated MDRD formula.[20] Micro-albuminuria was defined as a urinary albumin excretion of more than 30 mg/24h. Urinary osmolarity was calculated as [urinary osmolarity = 2 * (urinary sodium concentration + urinary potassium concentration) + urinary urea concentration].[21] This calculated osmolarity was not different from measured values (independent sample t-test p=0.94 in 100 samples, measured in our own lab).
Renal function measurements were performed using the constant infusion method with ^{125}I-Iothalamate and ^{131}I-hippuran.[22;23] Patients came non-fasting and were able to drink ad libitum but no caffeinated drinks. Antihypertensive medications were not withheld. Smoking was not

allowed during the measurement. After drawing a timepoint-0 blood sample, a priming solution containing 20 ml infusion solution (0.04 MBq of ^{125}I-Iothalamate and 0.03 MBq of ^{131}I Hippuran) is given at 08.00 hours, followed by a constant infusion of 6 ml/h to 12 ml/h, with lowest infusion rates in subjects with impaired renal function, based on previously known serum creatinine. Plasma concentrations of both tracers are allowed to stabilize during 1.5 hour equilibration, which is followed by two two-hour periods for simultaneous clearances of ^{125}I-Iothalamate and ^{131}I Hippuran. Clearances are calculated as (U * V/Piot and (I * V)/ Phipp respectively. As urinary clearance of ^{131}I-hippuran equals plasma clearance, in case of perfect urine collection, we routinely use the ratio of plasma-to-urinary clearance of ^{131}I-hippuran to correct urinary clearance of ^{125}I-Iothalamate for voiding errors24. This method to correct for urinary collection errors is extensively described and validated22-24. Coefficient of variation for GFR is 2.5% and for ERPF 5%.[22] Renal blood flow (RBF) was calculated as ERPF / (1-Hct). Renal vascular resistance (RVR) was calculated as MAP / RBF * 80,000.[16;25]
Patients underwent a standardized abdominal magnetic resonance imaging protocol without the use of i.v. contrast. Scanning was performed on a 1.5 Tesla MRI Magnetom Avento (Siemens, Erlangen, Germany) with the use of body matrix and spine matrix coils. T2 weighted fast imaging (true FISP) series were scanned during breath-hold. T2 weighted turbo spin echo (HASTE) series were scanned during free breathing with breath triggering on the diaphragm (prospective acquisition correction – PACE). Transversal images were obtained with fixed slice thickness of 5.0 mm. Coronal images were obtained with fixed slice thickness of 4.0 mm. Renal volume was measured on T2 weighted coronal images. Analyze Direct 8.0 (AnalyzeDirect, Inc., Overland Park, KS) software was used to analyse the volumes. Foxel size was forced to cubic to allow three dimensional viewing. Manual selection of the renal contours on every fifth slice with semi-automated propagation was used to obtain full selection of the kidneys, excluding the pyelum. All contours were checked manually before assessing total renal volume.

Statistical analyses

Analyses were performed with SPSS version 16.0 (SPSS Inc., Chicago, IL). Parametric variables are expressed as mean ± standard deviation (SD), whereas non-parametric variables are given as median (interquartile range). A two sided $p < 0.05$ was considered to indicate statistical significance. Differences between ADPKD patients and healthy controls were tested using an independent sample T-test when normally distributed, or a Mann-Whitney test when not normally distributed. Analyses were performed for ADPKD patients and healthy controls, divided into quartiles of age, illustrating the differences between patients and controls in the different stages of disease. P values for differences between the age quartiles were obtained using an ANOVA or a Kruskal-Wallis test. To investigate whether total renal volume was associated with GFR, ERPF, FF and RVR, multiple regression analysis was performed. Logarithmic transformation of total renal volume and of RVR was applied to fulfil the requirement of equal distribution of the residuals. Stepwise, various models were built to adjust for possible confounding. First these associations were investigated crude, and second adjusted for age and gender.

Results

A total of 103 ADPKD patients participated in this study; 60 (58%) were male. Mean age was 40 years. Table 1 depicts characteristics of ADPKD patients and of healthy controls. MAP was higher in the ADPKD group, despite frequent use of antihypertensive medication. Total renal volume was not measured in these healthy controls, but from literature it is known to be approximately 330 (194-614) ml.[26] Haemoglobin concentration was lower in ADPKD patients. Renal function, measured as clearance of iothalamate and estimated by the Modification of Diet in Renal Disease equation was also lower. In ADPKD patients, 24-hour urinary volume was 2.4±0.8 L, whereas this was 1.6±0.6 L in 103 age- and gender-matched subjects participating in the PREVEND-study (p<0.001). Urinary albumin excretion was also higher in ADPKD patients (42 (14-131) vs. 7(6-10) mg/24h), whereas calculated urinary osmolarity was lower (421±147 vs. 568±181 mOsm/l), both p<0.001).

Table 1 Characteristics of ADPKD patients (n=103) and of age- and gender matched healthy controls (live kidney donors, n=103)

Variable	*ADPKD patients*	*Healthy controls*	*p-value*
Age (y)	40 ± 11	40 ± 10	0.92
Male gender, n (%)	60 (58)	60 (58)	1.00
Body mass index (kg/m2)	26.1 ± 4.6	25.6 ± 3.8	0.47
Mean arterial pressure (mm Hg)	96 ± 9	92 ± 9	<0.001
Antihypertensive medication, n (%)	80 (78)	2 (2)	<0.001
Total renal volume (l)	1.49 (0.95-2.19)	-	-
Hb (mmol/l)	8.4 ± 0.8	8.9 ± 0.8	<0.001
Serum creatinine (umol/l)	111 ± 68	74 ± 11	<0.001
GFR (ml/min)	92 ± 36	124 ± 20	<0.001
ERPF (ml/min)	301 ± 125	475 ± 84	<0.001
FF	30 ± 4	26 ± 3	<0.001
RVR (dynes/cm^{-2})	16.1(11.6-21.8)	8.8 (7.9-1.0)	<0.001

Parametric variables are expressed as mean ± SD, whereas non-parametric variables are given as median (25th-75-th percentile). Abbreviations are: Hb, haemoglobin; eGFR, estimated Glomerular Filtration Rate; GFR, glomerular filtration rate; ERPF, effective renal plasma flow; FF, filtration fraction; RVR, renal vascular resistance.

For further analyses, ADPKD patients and healthy controls were subdivided into quartiles of age, as shown in Table 2. It shows the same variables as Table 1, but depicted per quartile of age, illustrating differences in variables under study between the different age categories. Variables that were different for the quartiles of ADPKD patients were use of antihypertensive medication, serum creatinine, (e)GFR, ERPF and RVR. Variables that were different for the quartiles of healthy controls were Hb, (e)GFR, ERPF, RVR, and FF.

Table 2 Characteristics of ADPKD patients and of age- and gender matched healthy controls (live kidney donors) per quartile of age

Variable	*Quartile 1*		*Quartile2*		*Quartile 3*		*Quartile 4*		*ADPKD*	*HC*
	ADPKD	**HC**	**ADPKD**	**HC**	**ADPKD**	**HC**	**ADPKD**	**HC**	**P for trend**	**P for trend**
Age (y)	27±5	28±5	35±2	36±2	43±3	43±3	54±5	54±5	<0.001	<0.001
Male gender, n (%)	16 (64)	16 (64)	16 (55)	16 (55)	12 (46)	12 (46)	16 (62)	16 (62)	0.6	0.6
BMI (kg/m2)	25.1±4.7	25.2±3.3	26.4±5.2	25.4±4.0	25.1±3.3	24.8±3.4	27.4±4.6	26.9±4.3	0.2	0.66
MAP (mm Hg)	96±11	91±12	96±7*	89±6	96±11	91±7	97±9	95±10	0.9	0.2
Antihyp med, n (%)	15 (60)*	1 (4)	23 (79)*	0 (0)	19 (73)*	0 (0)	24(92)*	1 (4)	0.05	1.0
ACEi/ARB, n (%)	14 (56)*	0 (0)	19 (73)*	0 (0)	18 (69)*	0 (0)	21 (81)*	0 (0)	0.3	1.0
Total renal volume (l)	1.0(0.7-2.0)	-	1.6(1.1-2.5)	-	1.6(1.1-2.1)	-	1.6(1.1-2.2)	-	0.1	-
Hb (mmol/l)	8.5±0.7*	9.0±0.8	8.3±0.8*	9.0±1.0	8.3±0.8	8.7±0.9	8.4±1.0*	8.9±0.8	0.9	0.04
Serum creat (umol/l)	93±46*	73±11	102±56*	74±10	107±63*	71±11	145±92*	78±13	0.04	0.2
GFR (ml/min)	117±32	129±17	102±30*	130±16	84±32*	122±24	74±47*	116±18	<0.001	0.04
ERPF (ml/min)	374±119*	527±83	335±103*	491±68	287±116*	454±88	203±104*	429±62	<0.001	<0.001
FF	32±4*	25±2	31±4*	26±3	29±4*	27±4	30±4*	27±2	0.1	0.007
RVR (dynes/cm^{-2})	12(10-16)*	8(7-9)	14(11-17)*	8(7-9)	17(13-21)*	10(8-12)	22(18-33)*	10(9-11)	<0.001	<0.001

Parametric variables are expressed as mean ± SD, whereas non-parametric variables are given as median (25th-75th percentile). Asterixes indicate a difference p<0.05 between ADPKD and healthy controls. P values at the right side of the table are obtained using ANOVA/Kruskal Wallis. Abbreviations are: ADPKD, autosomal dominant polycystic kidney disease; HC, healthy control; BMI, body mass index; MAP, mean arterial pressure; antihyp med, use of antihypertensive medication; Hb, haemoglobin; creat, creatinine; GFR, glomerular filtration rate; ERPF, effective renal plasma flow; FF, filtration fraction; RVR, renal vascular resistance.

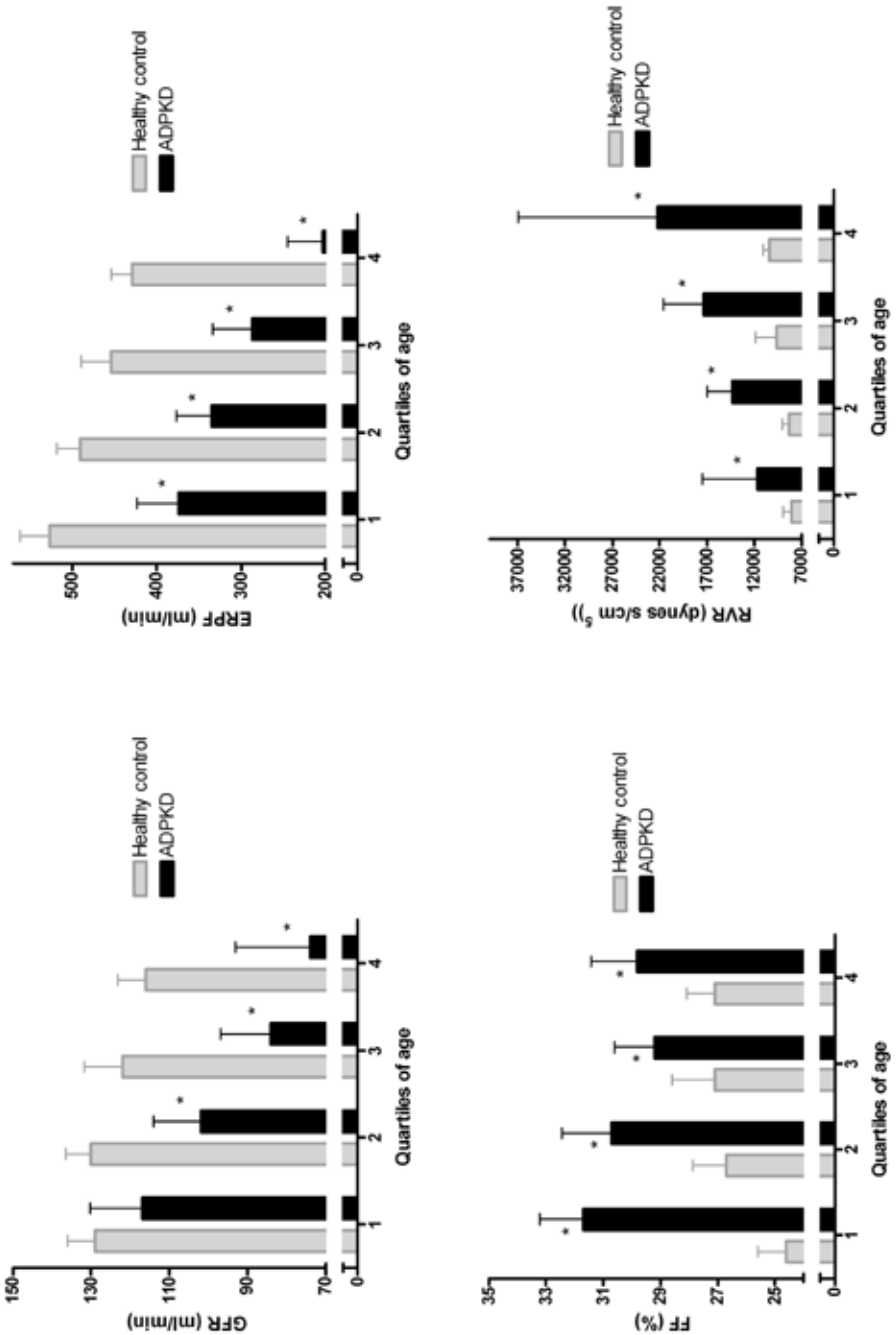


Figure 1: Renal hemodynamic parameters (mean values + 95% confidence intervals and for RVR median values and interquartile range) in ADPKD patients (n=103) and healthy controls (n=103). Asterixes indicate a p-value <0.05 compared with healthy controls in the same quartile of age.

Renal hemodynamic parameters for quartiles of age are depicted in Figure 1. GFR, ERPF, FF and RVR are shown per quartile of age for ADPKD patients and for healthy controls in the same graph (mean ± 95% confidence interval of the mean and for RVR median and interquartile range is shown). ERPF was decreased in ADPKD patients already in the first quartile of age, with a further decline with increasing age. In the youngest age quartile, GFR was not significantly different between ADPKD patients and healthy controls; consequently filtration fraction was considerably increased. In the older age cohorts, GFR decreased progressively in ADPKD, and much less steep in the healthy controls. Consequent to the parallel decreases in ERPF and GFR in ADPKD with increasing age, the elevated FF remained constant over the age quartiles, whereas in the healthy controls, FF increased with age. Within the healthy controls, BMI (R2 = 0.09, p=0.002) and age (R2 =0.07, p=0.008) were associated with FF, whereas these variables were not significantly associated with FF within the ADPKD patients (p=0.4 and 0.1, respectively). Within the ADPKD patients, FF was significantly associated with female gender (R2 = 0.05, p=0.02) and inversely with use of angiotensin converting enzyme inhibitors (ACEi) or angiotensin receptor blockers (ARB's) (R2 =0.07, p=0.01). Seventy-one of the 103 ADPKD patients used an ACEi/ARB; these patients had a lower FF than the patients who did not use an ACEi/ARB (30±4 vs. 32±4, p=0.01). Patients taking ACEi/ARB had the same Hb level as patients without these agents (p=0.45). As a consequence of the decreased RBF and the tendency towards a high MAP in ADPKD patients, renal vascular resistance (RVR) was high, with 12 (10-16) dynes s/cm² in the youngest quartile of age and 22 (18-33) dynes s/cm² in the oldest quartile.

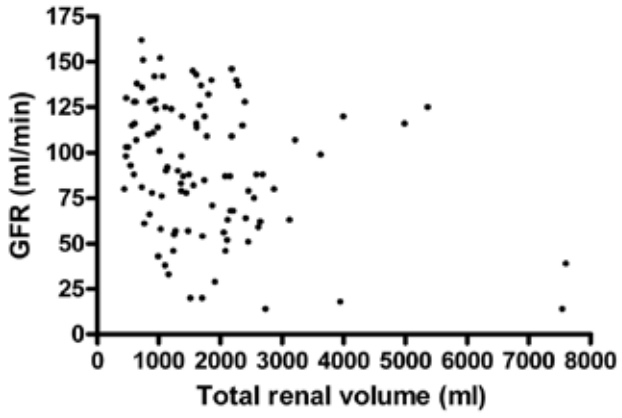
In ADPKD patients, total renal volume was related to GFR (standardized β(st β)= -0.31, p=0.002), ERPF (st β= -0.23, p=0.02), FF (st β=-0.33, p = 0.001) and RVR (st β=0.33, p = 0.001). After adjustment for age and gender, these associations did not change materially (Table 3). Although there was an association between total renal volume and GFR, there were several patients with large kidneys, but still a reasonable renal function and patients with relatively small kidneys, but already a markedly decreased renal function (Figure 2).

Table 3: Univariate and multivariate associations between total renal volume and renal hemodynamics for ADPKD patients only

variable	*model*	*St β*	*p*	R^2
GFR	1	-0.31	0.002	0.093
	2	-0.37	0.001	0.269
ERPF	1	-0.23	0.02	0.053
	2	-0.29	0.008	0.241
FF	1	-0.33	0.001	0.111
	2	-0.24	0.01	0.237
RVR	1	0.32	0.001	0.101
	2	0.38	<0.001	0.292

Standardized beta's and p values were calculated using multivariate linear regression. The R2 aplies to the whole model. Dependent variable is log transformed total renal volume. Independent variables are the above mentioned variables (GFR, ERPF, FF and log RVR).
Model 1: crude
Model 2: adjusted for age and gender
Abbreviations are: GFR, glomerular filtration rate; ERPF, effective renal plasma flow; FF, filtration fraction and RVR, renal vascular resistance.

Figure 2: Association between total renal volume and glomerular filtration rate (GFR) for participating ADPKD patients (n=103).



Urinary albumin excretion was elevated in the first quartile of age compared with healthy controls (70 (19 to 131) vs. 7(5 to 10) mg/24h). Sixty-eight percent of these patients in this age quartile were microalbuminuric, whereas 56% of the patients in this age quartile used ACEIs or ARBs. ADPKD patients with microalbuminuria had significantly larger kidneys than patients with normoalbuminuria (P<0.001). Of note, patients in the youngest quartile of age without microalbuminuria (n=8) already had a decreased ERPF and increased FF and RVR compared with healthy controls, while again GFR was again not different from healthy controls.
Twenty-four-hour urinary volume was higher and urinary osmolarity was lower in ADPKD patients versus healthy controls in all quartiles of age. In the first quartile, these values were: 2.1±0.9 vs. 1.4±0.5 l/24h (p=0.002), and 497±183 vs. 600±165 mOsm (p=0.04), respectively, and in the fourth quartile: 2.5±0.9 vs. 1.8±0.5 l/24h (p=0.002), and 367±113 vs. controls 516±167 mOsm/l (p=0.001). In ADPKD patients, urinary volume tended to increase with age, whereas urinary osmolarity decreased (p=0.003).

Discussion

In the study presented here, we found that young adult ADPKD patients (16 to 33 years) already had a markedly decreased ERPF when compared with healthy controls, despite an almost normal GFR and a modestly enlarged TRV. Because of the decreased ERPF and the tendency for a high MAP, young adult ADPKD patients had an increased RVR and an especially high FF. These abnormalities in ERPF and FF are an early phenomenon and remained present throughout the age quartiles. Also microalbuminuria was already present early in the disease. In the young adult ADPKD patients, 24-hour urinary volume was higher and 24-hour urinary osmolarity was lower compared with normal controls. Urinary volume tended to increase, whereas urinary osmolarity decreased over the age quartiles of ADPKD patients.
We found a slightly decreased GFR, accompanied by a much stronger decrease in ERPF, in young adult ADPKD patients. There are several possible explanations for this hemodynamic profile; for instance, that ADPKD is primarily a disease of the tubules and interstitium, and that extraction of hippuran could therefore be disturbed. However, the fact that we found the difference in these hemodynamic parameters in patients versus healthy controls, most pronounced in the young adult ADPKD subjects in whom tubular function and interstitium are still relatively preserved, suggests that this explanation is less likely. An alternative explanation for the observed renal hemodynamic profile could be a state of predominantly efferent renal vasoconstriction related to increased neurohumoral activation. Neurohumoral activation has been described to occur early in ADPKD,[27] and efferent renal vasoconstriction related to increased neurohumoral activation is characteristic of other hypertensive conditions.[28] We hypothesize that this hemodynamic profile indicates hyperfiltration. The loss of nephrons due to the disease process results in a decreased ERPF, whereas compensatory GFR goes up in the remnant nephrons. The presence of an elevated albuminuria already in the youngest quartile of ADPKD patients supports this hypothesis. The phenomenon of hyperfiltration could explain why GFR stays stable for a number of years and then rapidly declines.[9;10] This hypothesis also indicates that GFR is indeed not an appropriate marker for disease severity, as has been suggested10, and underlines the importance of a search for better markers of disease severity.

To the best of our knowledge, no previous studies looked directly at possible hyperfiltration in ADPKD patients. Two studies so far suggested that patients with ADPKD may hyperfiltrate. Dimitrakov et al suggested hyperfiltration on the basis of measurement of creatinine clearance and serum β2 microglobulin levels.[29] Another study described a high GFR in very young ADPKD patients (9.8 ± 5.9 y), as measured with a technetium 99m DTPA single-injection technique.[30] Some of the ADPKD patients in our study, have a GFR comparable to the values described in these studies (Figure 2). Importantly, in both studies no information was obtained on ERPF and FF and no comparison with healthy controls was made. This makes it less possible to draw firm conclusions whether there is indeed hyperfiltration in those patients. We found that FF was especially high in young adult ADPKD patients, whereas in healthy controls FF is high in the older age groups. The latter is in line with previous analyses, showing that higher BMI and older age are the main determinants of a higher FF.[31;32] These data, in combination with the fact that we found a negative correlation between total renal volume and filtration fraction (R=-0.28, p=0.004) suggests that hyperfiltration is a process that occurs early in the disease.

A high filtration fraction, reflecting elevated glomerular pressure, has been shown to contribute to progressive renal function loss in experimental studies.[33-36] Evidence in humans is sparse, but recently, in renal transplant recipients, a high FF was associated with increased graft loss, independent of blood pressure, GFR and proteinuria, which also supports a role for elevated glomerular pressure in human.37 The finding of a high FF early in ADPKD could provide a rationale for prescription of agents that inhibit the renin angiotensin aldosterone system (RAAS) early in the disease, because these agents are known to decrease FF and intraglomerular pressure.[38;39] As yet there is no evidence that inhibiting the RAAS is indeed beneficial in ADPKD. However, the studies that were performed to investigate the efficacy of RAAS intervention to preserve renal function in ADPKD patients may have been too underpowered and had a too short of follow-up to reach firm conclusions.[4;40] Of note, in our study, 71 of the 103 ADPKD patients used an ACEi/ARB, but the group still had a very high FF (although patients using an ACEi/ARB had a FF lower than the patients who did not use an ACEi/ARB (30±4 vs. 32±4, p=0.01)), supporting the fact that inhibiting the RAAS also results in a decreased FF in ADPKD patients. At the moment, a large-scale study is being performed with 4 years of follow-up in which the efficacy of complete RAAS blockade to preserve renal volume and renal function is investigated (HALT-PKD). Hopefully, this study will answer the question whether RAAS blockade is effective in preventing renal function decline in ADPKD.[41]

Our findings on renal vascular resistance, renal blood flow, decreased urine concentration and albuminuria are in line with data from the literature. Renal vascular resistance and renal blood flow have been measured in only two studies performed on the same cohort[15;16]. Renal blood flow was measured in a subgroup of 131 patients using magnetic resonance imaging. During follow-up, a reduction of renal blood flow and an increase in renal vascular resistance paralleled the increase in total kidney volume, and importantly, preceded GFR decline and predicted structural and functional disease progression in this cohort. This suggests that low RBF and high RVR could be good markers for rapid disease progression. However, these studies did not include a comparison with healthy subjects. We found renal vascular resistance to be elevated early in the disease compared with healthy controls.

In the first quartile of age, serum creatinine in ADPKD patients was significantly increased compared with healthy controls. The fact that GFR was not different, suggests that other factors than renal function play a role (e.g., muscle mass). Hb levels were also different in the first quartile of age. Unexpectedly, ADPKD patients had lower Hb values compared to healthy controls. There was no association between use of ACEi/ARB therapy and Hb level. Patients taking these agents had the same Hb as patients without these agents (p=0.45), so use of ACEIs/ARBs is not a likely explanation. An explanation for the difference in Hb could be a diminished erythropoietin production. Unfortunately, we do not have information on epo levels.

The increased 24-hour urinary volume and decreased 24-hour urinary osmolarity that we found in the ADPKD patients is consistent with the decreased concentrating capacity that has been described in these patients.[12] Also, albuminuria has been described to occur at early stages of the disease.[13] We also found a high prevalence of microalbuminuria in young adult ADPKD patients. In addition, we found a decreased ERPF and increased filtration fraction, compared with healthy controls, even in young patients with no microalbuminuria yet. This suggests that early renal and vascular changes are present before occurrence of microalbuminuria, making ERPF and RVR likely to be early markers of disease severity.

We acknowledge that this study has limitations. First, we studied age versus renal hemodynamic parameters in a cross-sectional study. In such a design, selection bias can occur. Also, patients were allowed to use their antihypertensive medication, potentially influencing renal hemodynamics. However, a life time longitudinal investigation of renal hemodynamic parameters is almost not feasible. Second, we did not control water intake. However, our patients drank at libitum and did not receive advice to deliberately increase water intake. We believe that urine concentrating capacity in ADPKD is indeed lower at increased age. The strengths of our study include that we simultaneously measured various renal hemodynamic parameters in a relatively large group of ADPKD subjects in various stages of the disease. The simultaneous measurement of GFR and ERPF using the gold standard (clearance of iothalamate and hippuran) is the best surrogate to study hyperfiltration. Previous studies investigating renal hemodynamics in ADPKD used only GFR measurements, from which it cannot be concluded whether hyperfiltration is present when there is no information on renal blood flow. Furthermore, we were able to compare ADPKD patients with age- and gender-matched healthy controls, enabling comparison with the normal situation.

In conclusion, already at young adult age, ADPKD patients have marked renal abnormalities, despite only modestly enlarged kidneys and a near-normal GFR. These data suggest that in ADPKD, a renal hemodynamic profile, characterized by decreased ERPF and increased FF may be an early sign indicating more outspoken disease severity. The finding furthermore indicates that ERPF and FF (in addition to renal volume and albuminuria) may indicate disease severity better than GFR.

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Part I

Chapter 3

Association of urinary biomarkers with disease severity in patients with Autosomal Dominant Polycystic Kidney Disease: A Cross-Sectional Analysis.

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Abstract

Disease monitoring of ADPKD will become more important with upcoming therapeutic potential interventions. Since serum creatinine is considered of limited use and measurement of effective renal blood flow (ERBF) and total renal volume (TRV) are time consuming and expensive, there is a need for other biomarkers. We aimed to investigate which urinary markers are elevated in ADPKD, whether these urinary markers are associated with measured glomerular filtration rate (mGFR), ERBF and TRV and whether these associations are independent of albuminuria (UAE).

We measured 24-h urinary excretion of albumin, of glomerular- (IgG), proximal tubular- (KIM1, NAG, NGAL, β2 microglobulin) and distal tubular (H-FABP) damage markers and inflammatory markers (MCP-1 and MIF) in 102 ADPKD patients and compared these to 102 age- and gender matched healthy controls. For ADPKD patients, disease severity was assessed by measures of function (mGFR and ERBF, measured by clearance of 125I-Iothalamate and 131I Hippuran, respectively, during continuous infusion) and structure (TRV, measured by MRI.

In 102 ADPKD patients (aged 40±11y, 58% male), all measured urinary biomarkers were elevated compared to healthy controls. Excretion of IgG and albumin were relatively most elevated. ERBF and mGFR were associated with urinary excretion of β2 microglobulin, NGAL and H-FABP, independent of UAE, while TRV was associated with KIM-1, NGAL and MCP-1 independent of UAE.

Our results suggest that markers for multiple parts of the nephron are elevated in ADPKD and that measurement of urinary β2 microglobulin, H-FABP, MCP-1 and especially NGAL could be of value in addition to UAE for determination of disease severity in ADPKD.

Introduction

Autosomal Dominant Polycystic Kidney Disease (ADPKD), the most common hereditary kidney disease is characterised by progressive cyst formation in the kidneys, leading to pain, hematuria and end stage renal disease. End stage renal disease usually occurs in the 4th-7th decade of life.[1] The clinical course of ADPKD is highly variable between families, but also within families.[2] Current treatment cannot prevent renal failure.[3;4] However, a better understanding of the pathophysiology of the disease identified promising candidate drugs for renal preservation.[5] Clinical trials have been initiated for vasopressin-2 receptor antagonists, long-acting somatostatin analogues and mTOR inhibitors.[6] With these upcoming therapeutic regimens, and the existing variation in disease course, monitoring disease severity will become more important.

Measurement of serum creatinine to assess estimated glomerular filtration rate (eGFR) is of limited use in determining disease severity in ADPKD, since it has been suggested that GFR remains relatively long stable due to compensatory hyperfiltration, while disease progresses.[7] Effective renal blood flow (ERBF) and total kidney volume have been proposed as better markers for disease severity, for they predict renal decline during follow-up[7-10] However, measurement of RBF and total kidney volume is time consuming and expensive. Therefore, interest has grown in urinary biomarkers. These biomarkers are relatively easy to obtain and inexpensive to measure. In renal diseases in general, urinary Kidney Injury Molecule-1 (KIM-1) and Neutrophil Gelatinase-Associated Lipocalin (NGAL) for example are well described markers and predictors of renal disease progression[11-13] and may be promising candidate markers in ADPKD. In ADPKD patients, a limited number of urinary biomarkers have been investigated, namely albuminuria, β2 microglobulin,[14;15] NGAL16 and Monocyte Chemotactic Protein-1 (MCP-1).[17;18] In general, the promising results obtained for these markers have not been corroborated by others, except for albuminuria. Several studies showed that in ADPKD, albuminuria is associated with increased renal volume,[19;20] mean arterial blood pressure, filtration fraction,[20] renal growth and slope of glomerular filtration rate.[9] Albuminuria is therefore a valuable biomarker indicating disease severity and predicting outcome. As such it has become a secondary endpoint in intervention studies in ADPKD.[21]

In normal physiology, albumin passes the glomerular filtration barrier only in trace amounts, after which it is reabsorbed in the proximal tubule.[22;23] In general, albuminuria is assumed to reflect predominantly glomerular endothelial damage. ADPKD however is a disease characterized predominantly by structural abnormalities of especially the distal tubules and collecting ducts, and by interstitial inflammation and fibrosis. In ADPKD, albuminuria might therefore also reflect (inflammatory) tubular damage.

Given this background, we investigated in a cross-sectional study, in a well phenotyped cohort of 102 ADPKD patients, (1) whether urinary biomarkers reflecting glomerular, proximal or distal tubular damage or inflammation are elevated when compared to healthy controls, (2) whether these elevated biomarkers are associated with current markers of disease severity in ADPKD (effective renal blood flow and glomerular filtration rate as functional measures and total renal volume as measure of structural change) and (3) whether these associations are independent of albuminuria.

Materials and Methods

Patients and healthy controls

One hundred and eighteen consecutive patients with ADPKD visiting our out-patient clinic meeting our in- and exclusion criteria were asked to participate. Diagnosis of ADPKD was made upon Ravine criteria.[24] Subjects were considered ineligible to participate if they received renal replacement therapy (including renal transplantation), had undergone renal surgery, were unable to undergo magnetic resonance imaging (as having distorting foreign bodies or aneurysmal clips), had other systemic diseases potentially affecting renal function (such as diabetes mellitus), had other specific medical conditions such as pregnancy, lactation, or who were less than 6 months postpartum. After screening, subjects underwent an extensive medical history assessment and were scheduled for a 1-day outpatient clinic evaluation.
For this study, healthy controls were also invited to participate. These control subjects were matched for age and gender and were considered healthy in case they had a history without cardiovascular and/or renal disease, used no medication, had a normal blood pressure (systolic blood pressure < 140 and diastolic blood pressure <90 mmHg) and a normal estimated glomerular filtration rate (>60 ml/min *1.73 m2). This study was performed in adherence to the declaration of Helsinki. All subjects gave written informed consent.

Measurements and storage of urinary and plasma samples

Blood pressure was assessed with an automatic device (Dinamap) for 15 minutes during the renal function measurement. Systolic- and diastolic blood pressure values were used to calculate mean arterial pressure (MAP) using the standard formula: 2/3 diastolic blood pressure + 1/3 systolic blood pressure. Weight and height were determined. Body mass index (BMI) was calculated as weight in kilograms (kg) divided by height in square meters.
Patients collected a 24-h urine sample prior to the outpatient visit. Urinary albumin concentration was determined by immunonephelometry (BNII; Dade Behring Diagnostics, www.dadebehring.com). Urine was stored at -80°C until measurement of Immunoglobulin G (IgG), Kidney Injury Molecule 1 (KIM1), N-acetyl-β-D-glucosaminidase (NAG), Neutrophil Gelatinase-Associated Lipocalin (NGAL), β2 microglobulin, Heart-type Fatty Acid Binding Protein (H-FABP), macrophage migration inhibitory factor (MIF) and monocyte chemotactic protein-1 (MCP-1). Urinary IgG is a marker for glomerular damage, urinary β2 microglobulin, KIM, NAG and NGAL are markers for damage of the proximal tubule,[12;25;26] whereas urinary H-FABP is a damage marker of the distal tubule.[27;28] Urinary MIF and MCP-1 are markers for inflammation.[29-32] Urinary IgG, HFAB-P (Hytest, www.hytest.fi), β2 microglobulin (Anogen, www.yesbiotech.com), KIM1, NGAL, MCP-1 and MIF (R&D systems, www.rndsystems.com) were measured by ELISA. Before measurement, urine samples were diluted two times for KIM-1, β2 microglobulin, MCP1 and MIF, 5 times for H-FABP, and 100 times for NGAL and IgG.
Detection limit for KIM-1 was 0.087 ng/ml, for IgG 220 ng/ml, for NAG 22 ng/ml, for MCP 0.04 ng/ml, for MIF 0.06 ng/ml, for β2 microglobulin 18 ng/ml and for H-FABP 0.38 ng/ml. Urinary N-acetyl-β-D-glucosaminidase (NAG) was measured using a modified enzyme assay according to Lockwood and corrected for nonspecific conversion (HaemoScan, www.haemoscan.com). For all urinary markers, urinary excretion was calculated by multiplication with the volume of the 24-hour urine collection, resulting in biomarker excretion expressed per 24 hours.
Prior to renal function measurement, blood samples were drawn for determination of haemoglobin, glucose, creatinine and urea. Concentrations of haemoglobin and glucose were measured in serum using standard methods. Creatinine was measured with the Roche enzymatic creatinine assay (IDMS traceable). Creatinine values were used to calculate an estimated glomerular filtration rate (eGFR), using the 4-variable MDRD formula.[33] Urinary creatinine excretion was normalized to body weight by dividing the 24h urinary creatinine excretion by the patient's weight in kilograms.
Renal function measurements were performed using the constant infusion method with 125I-Iothalamate and 131I-hippuran.[34-37] Effective renal blood flow (ERBF) was calculated as ERPF / (1-Hct). (Hematocrit was measured halfway during the renal function measurement.) Patients underwent a standardized abdominal magnetic resonance imaging protocol without the use of i.v. contrast. Scanning was performed on a 1.5 Tesla MRI Magnetom Avento (Siemens, Erlangen, Germany) with the use of body matrix and spine matrix coils. Renal volume was measured on T2 weighted coronal images38 (slice-thickness 4.0 mm) using Analyze Direct 8.0 (Analyze-Direct, Inc., Overland Park, KS) software.

Statistical analyses

Analyses were performed with SPSS version 16.0 (SPSS Inc., Chicago, IL). Normally distributed variables are expressed as mean ± standard deviation (SD), whereas non-normally distributed variables are given as median (interquartile range). A two sided $p < 0.05$ was considered to indicate statistical significance.
Differences between ADPKD patients and healthy controls were tested using the two-sample T-test when normally distributed, or Mann-Whitney test when not normally distributed. Secondary, we compared biomarker excretions for ADPKD patients with a relatively well preserved renal function (eGFR >60) with healthy controls.
To investigate whether biomarker excretions correlated with mGFR, ERBF and total renal volume, Spearman correlation coefficients were calculated. We also analyzed the reference test as dichotomous variables to calculate Reciever Operating Characteristics curves. As cut-off values an ERBF <500 ml/min, a mGFR <60 ml/min and a TRV > 1000 mL were used. To be able to adjust for potential confounders, multiple regression analysis was performed. Biomarkers were log base 2 transformed to fulfil the requirement of normal distribution of the residuals. Two models were built. First, the association between biomarkers and variables of interest was investigated adjusted for age and gender, and second, albuminuria was entered as an independent variable in addition to the previous variables to investigate whether the associations were independent of albuminuria. To check for co-linearity, variance inflation factor diagnostic was used. The VIF diagnostic is the reciprocal of tolerance (1-R squared for the regression of that variable on all the other independents, ignoring the dependent). So when VIF is high, there is high multicollinearity and instability of the beta coefficients. To visualise the independency of albuminuria in the associations of urinary biomarkers with glomerular filtration rate, renal

blood flow and total renal volume, figures were made. For these figures, the excretion of various biomarkers was divided into tertiles. Linear regression analysis was used to calculate adjusted values of measured glomerular filtration rate and effective renal blood flow and geometric mean total renal volume (all ± 95% CI of the mean) per tertile. Means were adjusted for age, gender and urinary albumin excretion. P values for the unadjusted tertiles are calculated using ANOVA. As sensitivity analyses, we attempted to adjust for potential errors in 24h urine collection all analyses by repeating all analyses using biomarker/creatinine ratios as the index test (instead of 24h urinary excretions). We also repeated the analyses in which we only included ADPKD subjects with a mGFR > 60 mL/min.

Results

Thirteen patients refused to participate, two patients were not eligible to participate and 1 patient did not collect 24-h urine, leaving 102 patients for analyses. Characteristics of the participating patients and healthy controls are presented in table 1. A total of 102 ADPKD patients (58% male, aged 40±11 years) and 102 healthy controls were analysed. As shown in table 1, ADPKD patients had a higher body mass index, blood pressure (despite more frequent use of antihypertensive medication), serum creatinine, and 24-h urinary volume than age-and gender matched healthy controls. Haemoglobin and estimated glomerular filtration rate were lower than in healthy controls.

Table 1: Characteristics of 102 ADPKD patients and 102 age- and gender matched healthy

Variable	*ADPKD*	*Healthy controls*	*p-value*
Age (y)	40 ± 11	39 ± 12	0.5
Male, n (%)	59 (58)	59 (58)	1.0
BMI (kg/ m^2)	26±5	23±3	<0.001
BSA (m^2)	2.1±0.3	1.9 ± 0.2	<0.001
SBP (mm Hg)	129 ± 12	122 ± 12	<0.001
DBP (mm Hg)	80 ± 9	72 ± 8	<0.001
Antihypertensive medication, n (%)	78 (77)	0 (0)	<0.001
Hb (g/l)	135 ± 14	140 ± 13	0.003
Plasma creatinine (mg/dl)	1.3 ± 0.8	0.8 ± 0.1	<0.001
24h urinary volume (l/24h)	2.3 ± 0.8	2.0 ± 0.8	0.003
eGFR(ml/min per1.73 m^2)	68 ± 27	92 ± 12	<0.001
mGFR (ml/min)	91 ± 36	-	-
mGFR/BSA (ml/min/1.73 m^2)	77 ± 31	-	-
RBF (ml/min)	498 ± 207	-	-
Total renal volume (l)	1.5 (0.9-2.2)	-	-

Variables are presented as mean ± SD. Significance was tested using the two-sample T-test. Abbreviations are: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; Hb haemoglobin; eGFR, estimated glomerular filtration rate; mGFR, measured glomerular filtration rate; RBF, renal blood flow.

Association of urinary biomarkers with ADPKD

To answer our first research question, we investigated urinary biomarkers for different segments of the nephron in ADPKD patients and in healthy controls. Urinary creatinine excretion normalized to body weight was 19.3 ± 5.6 mg/kg for the whole group.

Table 2: Urinary biomarker excretions for ADPKD patients and controls

Variable	*ADPKD*	*Controls*	*p-value*
• UAE (mg/24u)	41.2 (14.9-121.7)	7.8 (6.4-10.4)	<0.001
Glomerular			
• IgG (mg/24u)	15.3 (4.3-47.5)	0.2 (0.2-0.3)	<0.001
Proximal Tubular			
• β2 MG (μg/24u)	192.1 (77.6-589.1)	79.4 (49.1-121.3)	<0.001
• KIM-1 (μg/24u)	1.5 (1.0-2.2)	0.8 (0.4-1.2)	<0.001
• NAG (U/24u)	3.1 (0.8-8.1)	0.4 (0.3-2.4)	<0.001
• NGAL (μg/24u)	73.7 (29.2-158.2)	23.4 (16.5-30.8)	<0.001
Distal Tubular			
• H-FABP (μg/24u)	1.9 (1.3-3.2)	1.4 (1.0-2.2)	0.003
Inflammatory			
• MIF (μg/24u)	0.2 (0.1-0.3)	0.1 (0.1-0.2)	0.03
• MCP (μg/24u)	0.7 (0.6-1.1)	0.3 (0.2-0.4)	<0.001

Variables are presented as median (25th percentile-75th percentile). Significance was tested using the Mann-Whitney U test. Abbreviations are: UAE, urinary albumin excretion; IgG, immunoglobulin G; β2 MG, β2 microglobulin; KIM-1, Kidney Injury Molecule 1; NAG, N-acetyl- β-D-glucosaminidase; NGAL, Neutrophil Gelatinase-Associated Lipocalin; H-FABP, Heart-type Fatty Acid Binding Protein; MIF, macrophage migration inhibitory factor; MCP-1, monocyte chemotactic protein-1.

Table 2 shows that albuminuria, glomerular, proximal- and distal tubular damage markers, as well as markers reflecting inflammation, were all significantly elevated in ADPKD patients compared to healthy controls. IgG excretion and albuminuria were relatively more elevated (6-77 x) in the ADPKD patients than excretion of the other markers (1.4-7.7 x). Values of the urinary markers for ADPKD patients and healthy controls are depicted in box plots in Figure 1. Although significantly different, there is some overlap in biomarker excretions between the ADPKD patients and healthy controls. To adjust for potential errors in 24h urine collection, also marker/creatinine ratios were compared. All urinary biomarker/creatinine ratio's, except MIF, remained significantly higher in ADPKD patients compared to healthy controls. When patients with a reasonably well preserved renal function (eGFR >60 ml/min per 1.73 m2, n=60) were selected, all urinary markers (except for H-FABP) were still significantly elevated compared to healthy controls. Also, when patients were selected with a relatively well preserved renal blood flow (ERBF>500 ml/min, n=45) or with a relatively modest total renal volume (TRV <1000 ml, n=30), all urinary markers (except for H-FABP and MIF) were elevated compared to healthy controls.

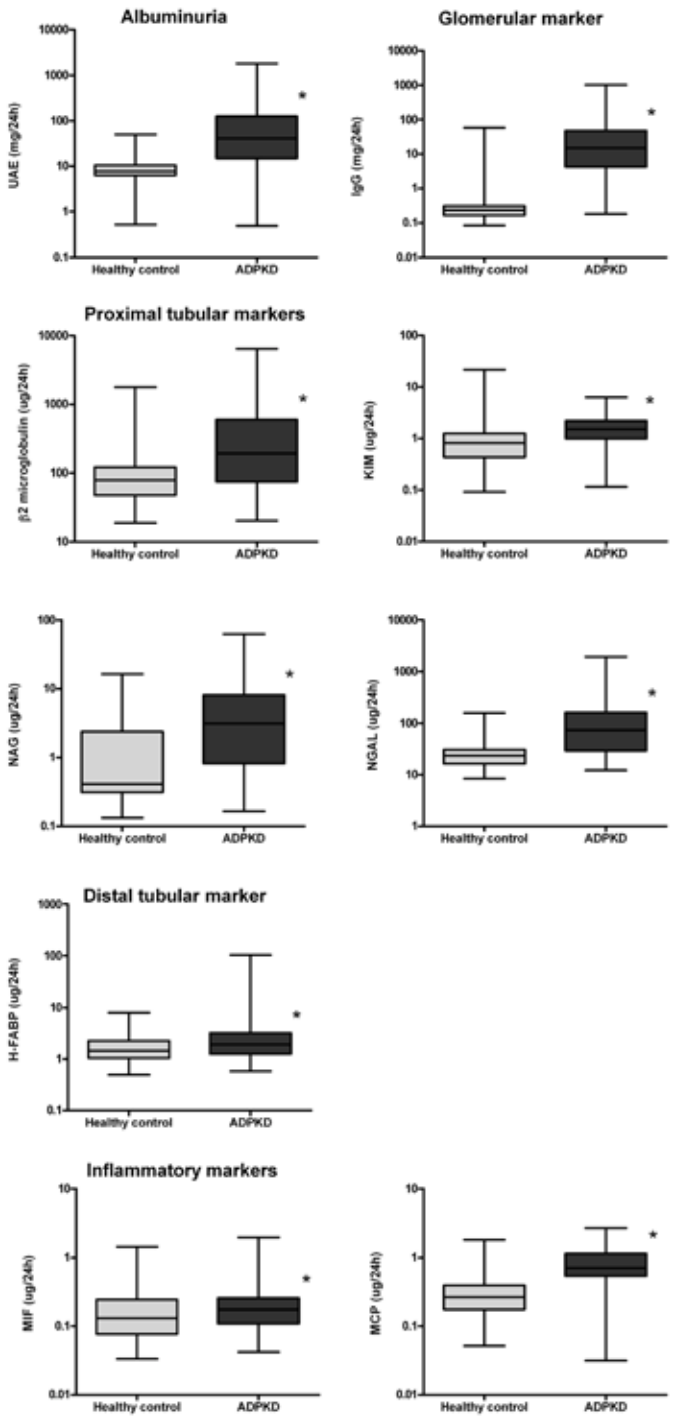


Figure 1: Urinary biomarkers for ADPKD patients and healthy controls. Boxplots with whiskers from minimum to maximum.

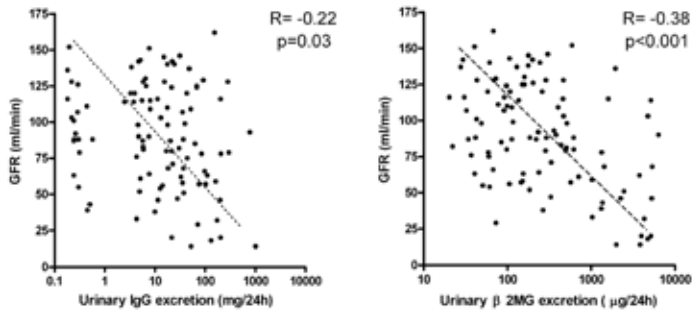
Association of urinary biomarkers with functional measures in ADPKD

Glomerular filtration rate and effective renal blood flow correlated strongly with each other (R=0.94, p<0.001). The associations of urinary markers with measured glomerular filtration rate (mGFR) and effective renal blood flow are depicted in table 3. Figures 2 and 3 depict scatterplots of the associations in table 3 with a p-value below 0.05. These are scatterplots for excretion of IgG, β2 microglobulin, NAG, NGAL, H-FABP and MCP-1 with mGFR (Figure 2), and IgG, β2 microglobulin, NAG, NGAL, H-FABP and MCP-1 with ERBF (Figure 3). For measured glomerular filtration rate and effective renal blood flow, the strongest association was with NGAL. Furthermore, IgG correlated strongly with UAE (R=0.70, p<0.001, not depicted in the table).

Table 3: Crude correlation coefficients of urinary biomarkers with glomerular filtration rate, renal blood flow and total renal volume in ADPKD patients.

Variable	*Glomerular Filtration Rate*		*Renal Blood Flow*		*Total Renal Volume*	
	R	p-value	R	p-value	R	p-value
Glomerular						
• IgG	-0.22	0.03	-0.21	0.04	0.35	<0.001
Proximal Tubular						
• β2 MG	-0.38	<0.001	-0.37	<0.001	0.14	0.2
• KIM-1	0.09	0.4	0.05	0.6	0.23	0.02
• NAG	-0.30	0.002	-0.29	0.004	0.27	0.007
• NGAL	-0.43	<0.001	-0.45	<0.001	0.16	0.1
Distal Tubular						
• H-FABP	-0.37	<0.001	-0.36	<0.001	0.13	0.2
Inflammatory						
• MIF	0.04	0.7	0.03	0.8	0.03	0.8
• MCP-1	-0.23	0.02	-0.20	0.05	0.58	<0.001

Correlations and significance were calculated using the spearman correlation coefficient. Abbreviations are: IgG, immunoglobulin G; β2 MG, β2 microglobulin; KIM-1, Kidney Injury Molecule 1; NAG, N-acetyl-β-D-glucosaminidase; NGAL, Neutrophil Gelatinase-Associated Lipocalin; H-FABP, Heart-type Fatty Acid Binding Protein; MIF, macrophage migration inhibitory factor; MCP-1, monocyte chemotactic protein-1.



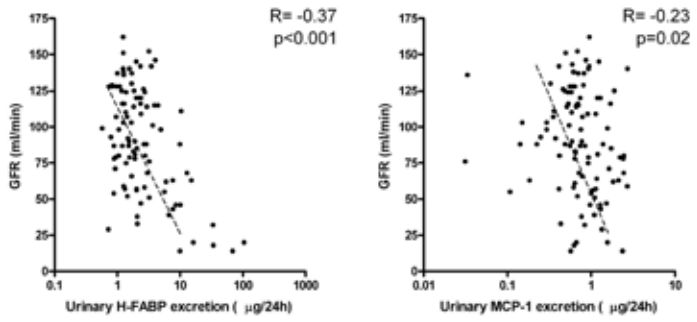
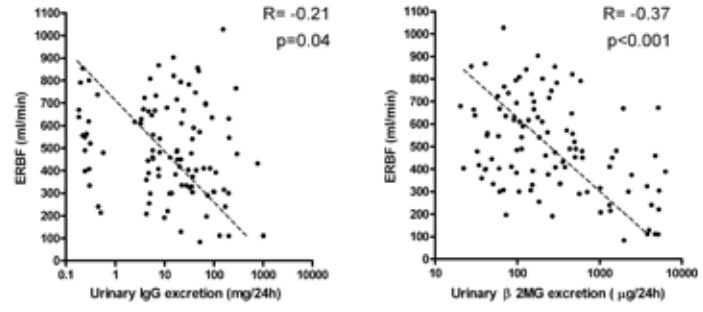


Figure 2: Associations between urinary biomarkers and Glomerular Filtration Rate.
Depicted are graphs of the urinary biomarkers under study that were significantly associated with mGFR (table 3).



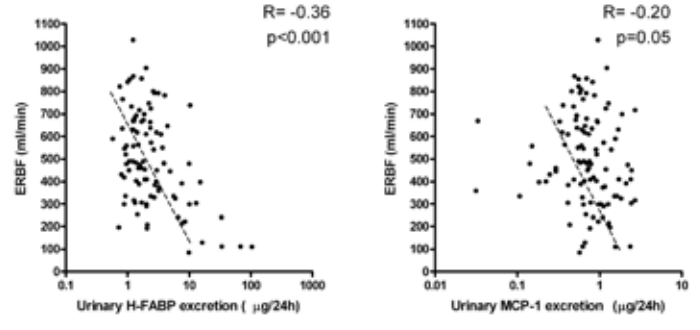
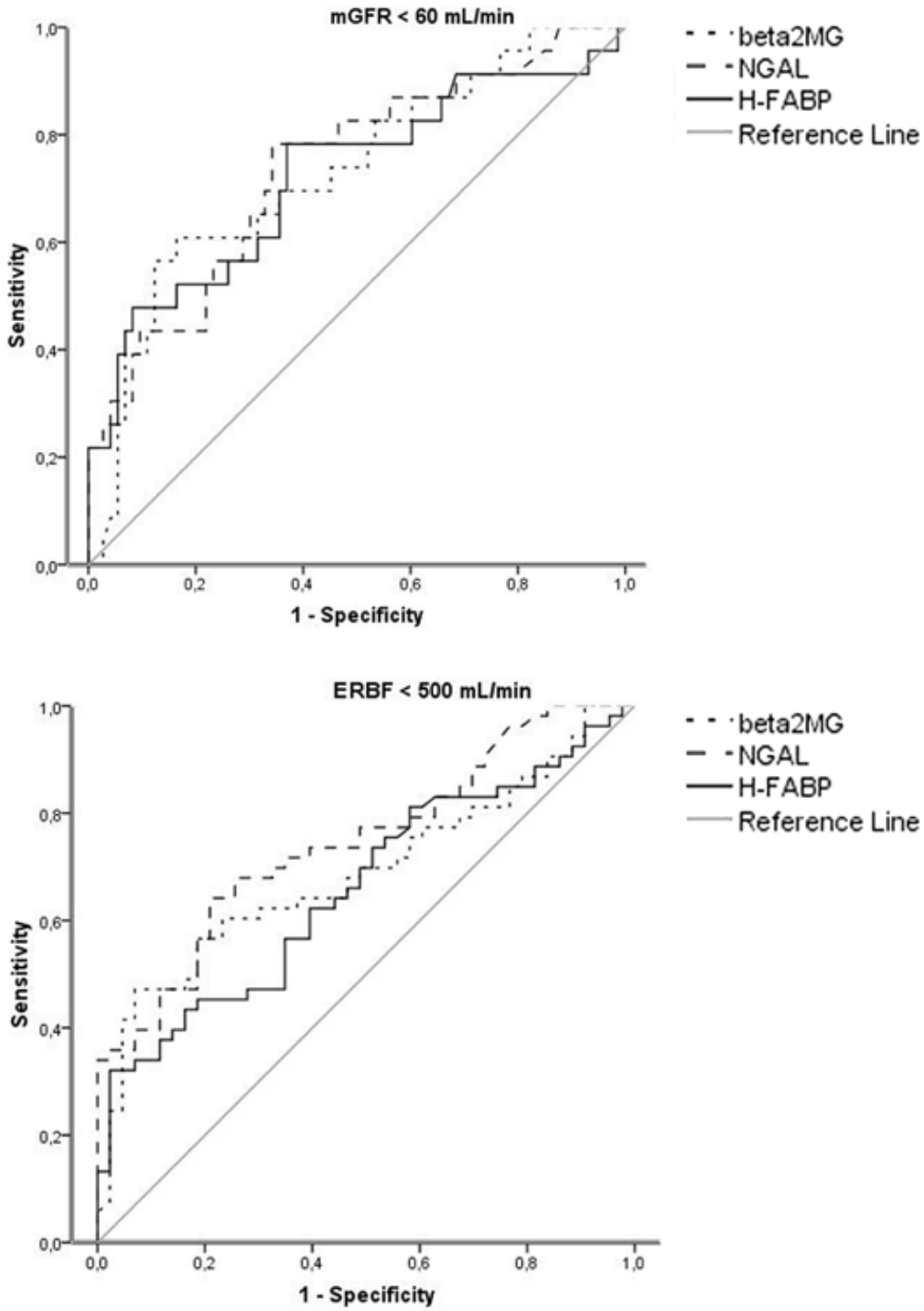


Figure 3: Associations between urinary biomarkers and Effective Renal Blood Flow (ERBF). Depicted are graphs of the urinary biomarkers under study that were significantly associated with ERBF (table 3).

ROC curves for an ERBF < 500 ml/min and a mGFR <60 ml/min are depicted in Figure 4 for the urinary biomarkers with the highest R values. All area's under the curves of the depicted urinary biomarkers are significantly different from chance. For both functional measures, NGAL has the largest AUC (0.74 and 0.75 respectively).



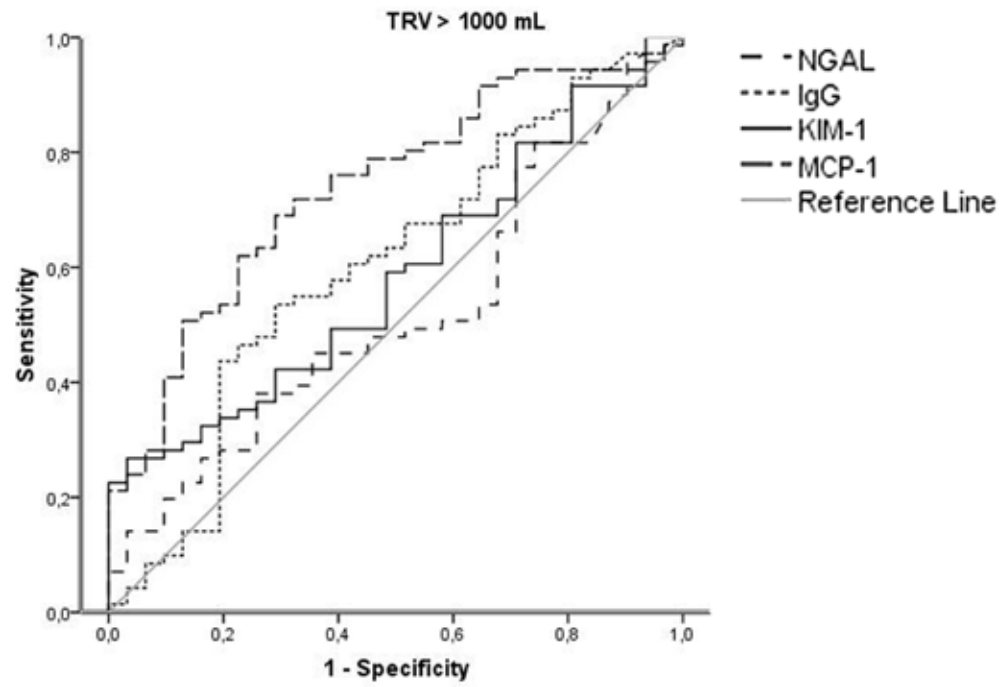


Figure 4: Reciever Operating Characteristics curves for various urinary biomarkers for dichotomized mGFR (cut-off <60 ml/min), ERBF (cut-off 500 ml/min) and TRV (cut-off >1000 mL).

Our third research question was whether these potential associations were independent of albuminuria (urinary albumin excretion, UAE). Table 4 shows the associations between 24h urinary biomarker excretions with measured glomerular filtration rate and Table 5 with effective renal blood flow, both adjusted for age, gender and albuminuria. The urinary biomarker excretions are log base 2 transformed. The beta therefore represents the change in mGFR or RBF per doubling of the biomarker. Of note, albuminuria (adjusted for age and gender) correlated well with both measured glomerular filtration rate and with effective renal blood flow. There are several associations that remained significant after adjustment for age, gender and albuminuria. For both measured glomerular filtration rate and for effective renal blood flow, β2 microglobulin, NGAL and H-FABP remained significantly associated. Figures 5 and 6 depict the associations between biomarkers that were associated with measured glomerular filtration rate independent of albuminuria (Figure 5) and effective renal blood flow independent of albuminuria (Figure 6). The figures show mean values of measured glomerular filtration rate and effective renal blood flow for tertiles of the aforementioned biomarkers, both crude and after adjustment of age, gender and albuminuria.

Table 4: Multivariable associations of various biomarkers with glomerular filtration rate after adjustment for age, gender (model 1) and additional adjustment for UAE (model 2).

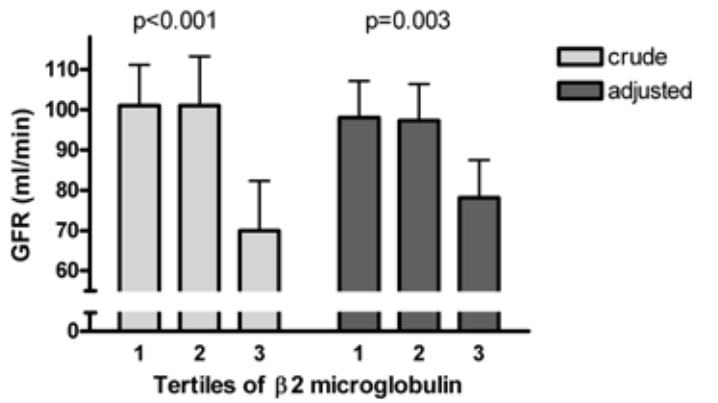
	model	*Urinary biomarker*			*UAE*		
		β	95% CI for β	p-value	β	95% CI for β	p-value
• UAE	1	-	-	-	-6.1	-8.9- -3.2	<0.001
Glomerular							
• IgG	1	-3.2	-5.0- -1.5	<0.001	-	-	-
	2	-1.4	-3.7-0.9	0.22	-4.5	-8.3 - -0.8	0.02
Proximal tubular							
• KIM-1	1	2.0	-3.6-7.6	0.48	-	-	-
	2	5.2	-0.07-10.5	0.053	-6.8	-9.7—3.9	<0.001
• B2MG	1	-6.0	-8.4 - -3.6	<0.001	-	-	-
	2	-4.8	-7.2- -2.3	<0.001	-4.2	-7.1- -1.4	0.004
• NAG	1	-3.3	-6.4- -0.2	0.04	-	-	-
	2	-1.5	-4.5-1.6	0.35	-5.6	-8.6- -2.6	<0.001
• NGAL	1	-10.6	-14.0- -7.3	<0.001	-	-	-
	2	-8.9	-12.7- -5.2	<0.001	-2.8	-5.7-0.2	0.07
Distal tubular							
• H-FABP	1	-9.3	-13.5- -5.1	<0.001	-	-	-
	2	-7.3	-11.5- -3.1	0.001	-4.6	-7.6- -1.6	0.003
Inflammatory							
• MIF	1	-0.1	-5.4-5.1	0.9	-	-	-
	2	2.3	-2.6-7.3	0.4	-6.4	-9.3- -3.5	<0.001
• MCP-1	1	-7.5	-12.6- -2.5	0.004	-	-	-
	2	-2.6	-8.4-3.2	0.4	-5.2	-8.6- -1.8	0.003

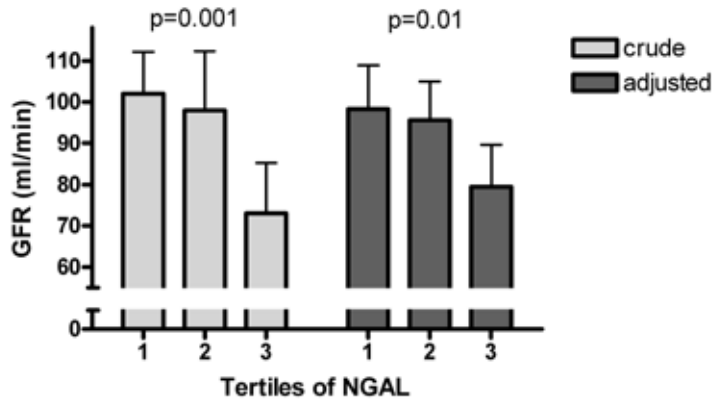
Beta's, confidence intervals and p-values were calculated using multivariable linear regression. Dependent variable is mGFR, independent variables are the log base2 transformed 24h excretions of the various urinary biomarkers.
Model 1: adjusted for age and gender
Model 2: adjusted for age, gender and albuminuria.
Abbreviations are: IgG, immunoglobulin G; β2 MG, β2 microglobulin; KIM-1, Kidney Injury Molecule 1; NAG, N-acetyl-β-D-glucosaminidase; NGAL, Neutrophil Gelatinase-Associated Lipocalin; H-FABP, Heart-type Fatty Acid Binding Protein; MIF, macrophage migration inhibitory factor; MCP-1, monocyte chemotactic protein-1.

Table 5: Multivariable associations of various biomarkers with effective renal blood flow after adjustment for age, gender (model 1) and additional adjustment for UAE (model 2).

	model	*Urinary biomarker*			*UAE*		
		β	95% CI for β	p-value	β	95% CI for β	p-value
• UAE	1	-	-	-	-30	-47- -13	0.001
Glomerular							
• IgG	1	-17	-27- -7	0.001	-	-	-
	2	-9	-23-4	0.18	-20	-42-2	0.08
Proximal tubular							
• KIM-1	1	4	-28-37	0.8	-	-	-
	2	19	-12-51	0.2	-32	-50- -15	<0.001
• B2MG	1	-34	-48 - -20	<0.001	-	-	-
	2	-28	-43- -14	<0.001	-19	-35- -2	0.03
• NAG	1	-21	-39- -3	0.02	-	-	-
	2	-13	-31-5	0.17	-26	-43- -8	0.005
• NGAL	1	-55	-75- -35	<0.001	-	-	-
	2	-47	-70- -24	<0.001	-12	-30-6	0.18
Distal tubular							
• H-FABP	1	-52	-77- -28	<0.001	-	-	-
	2	-43	-68- -18	0.001	-22	-39- -4	0.02
Inflammatory							
• MIF	1	-3	-33-28	0.9	-	-	-
	2	9	-20-39	0.5	-31	-48- -13	0.001
• MCP-1	1	-35	-65- -6	0.02	-	-	-
	2	-11	-45-23	0.5	-26	-46- -6	0.01

Beta's, confidence intervals and p-values were calculated using multivariable linear regression. Dependent variable is ERBF, independent variables are the log base2 transformed 24h excretions of the various urinary biomarkers.
Model 1: adjusted for age and gender
Model 2: adjusted for age, gender and albuminuria.
Abbreviations are: IgG, immunoglobulin G; β2 MG, β2 microglobulin; KIM-1, Kidney Injury Molecule 1; NAG, N-acetyl-β-D-glucosaminidase; NGAL, Neutrophil Gelatinase-Associated Lipocalin; H-FABP, Heart-type Fatty Acid Binding Protein; MIF, macrophage migration inhibitory factor; MCP-1, monocyte chemotactic protein-1.





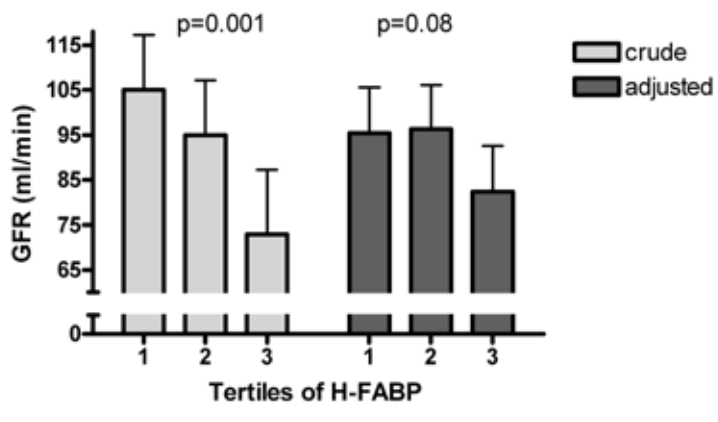
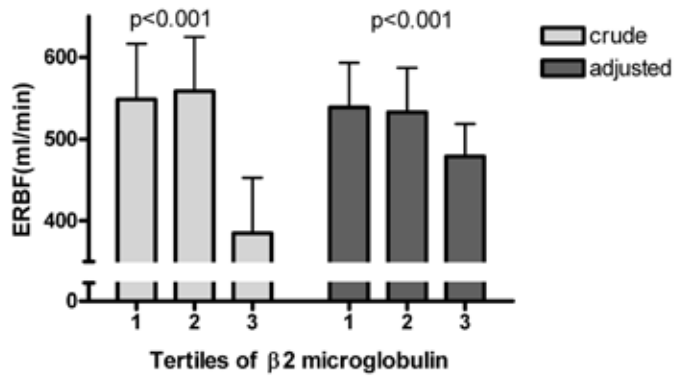
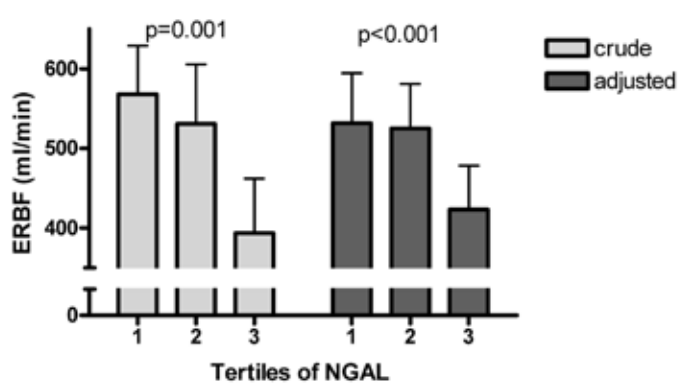


Figure 5: The associations between β2 microglobulin (upper panel), NGAL (middle panel) and H-FABP (lower panel), and mGFR are independent of albuminuria. Tertiles of the biomarkers are shown crude (light grey) and after adjustment of age, gender and albuminuria (dark grey).





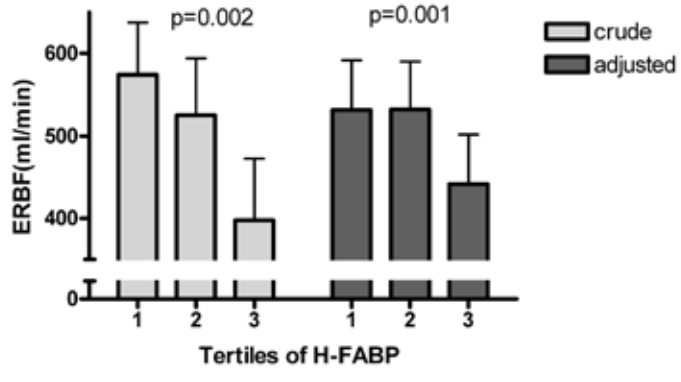


Figure 6: The associations between β2 microglobulin (upper panel), NGAL (middle panel) and H-FABP (lower panel), and ERBF are independent of albuminuria. Tertiles of the biomarkers are shown crude (light grey) and after adjustment of age, gender and albuminuria (dark grey).

Association of urinary biomarkers with structural changes in ADPKD

Effective renal blood flow (ERBF) and glomerular filtration rate (mGFR) correlated with total renal volume (TRV) (R=-0.20, p=0.05 and R=-0.28, p=0.004 respectively). Crude associations between the urinary biomarker excretions and total renal volume are depicted in table 3. Figure 7 consists of 4 scatterplots showing the association between IgG, KIM-1, NAG and MCP-1 with TRV. The strongest association for total renal volume was with MCP-1 excretion. The ROC curve in Figure 4 for a total renal volume < 1000 mL shows that MCP-1 has the largest AUC (0.73).

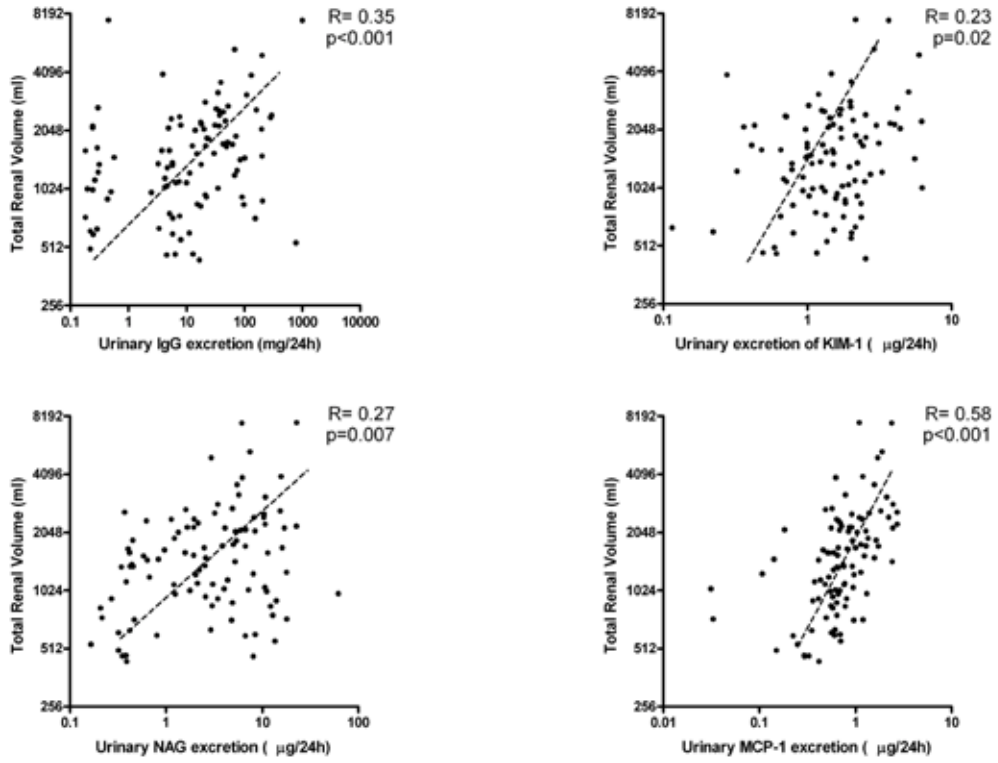


Figure 7: Associations between urinary biomarkers and Total Renal Volume.
Depicted are graphs of the urinary biomarkers under study that were significantly associated with TRV (table 3).

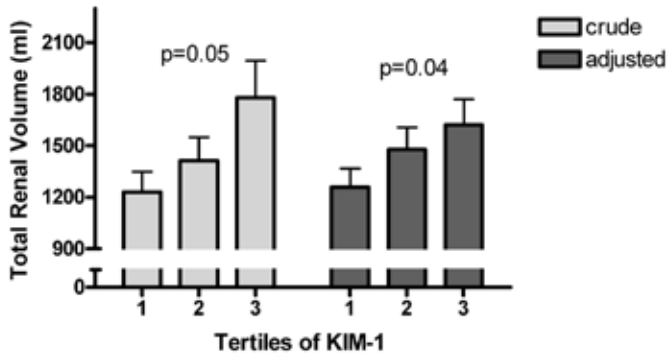
To investigate whether the potential associations were independent of albuminuria (urinary albumin excretion, UAE), Table 6 shows the associations between 24h urinary biomarker excretions and total renal volume adjusted for age, gender and albuminuria. KIM-1, NGAL and MCP-1 remained associated with total renal volume.

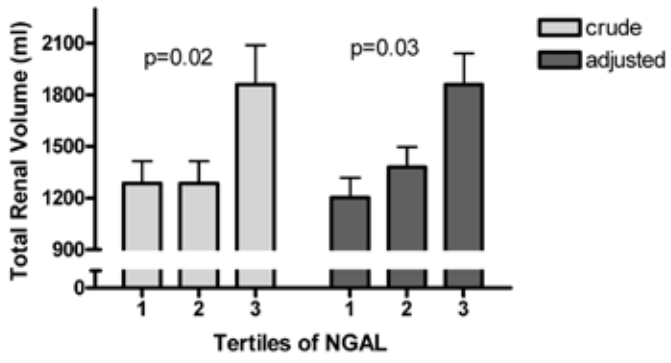
Figure 8 depicts the associations between the biomarkers that were associated with total renal volume independent of albuminuria. The figures show geometric mean values of total renal volume for tertiles of the aforementioned biomarkers, both crude and after adjustment of age, gender and albuminuria. Thus, NGAL remained associated with measured GFR, effective renal blood flow and total renal volume; all other biomarkers are associated with either mGFR / effective renal blood flow or total renal volume.

Table 6: Multivariable associations of various biomarkers with total renal volume after adjustment for age, gender (model 1) and additional adjustment for UAE (model 2).

	model	*Urinary biomarker*			*UAE*		
		β	95% CI for β	p-value	β	95% CI for β	p-value
• UAE	1	-	-	-	0.21	0.14-0.29	<0.001
Glomerular							
• IgG	1	0.10	0.05-0.14	0.001	-	-	-
	2	0.02	-0.04-0.08	0.5	0.19	0.09-0.29	<0.001
Proximal tubular							
• KIM-1	1	0.23	0.09-0.38	0.002	-	-	-
	2	0.15	0.01-0.28	0.04	0.19	0.12-0.27	<0.001
• β2MG	1	0.05	-0.02-0.13	0.15	-	-	-
	2	-0.01	-0.08-0.06	0.8	0.22	0.14-0.29	<0.001
• NAG	1	0.09	0.01-0.18	0.04	-	-	-
	2	0.03	-0.05-0.11	0.5	0.20	0.13-0.28	<0.001
• NGAL	1	0.22	0.12-0.32	<0.001	-	-	-
	2	0.12	0.02-0.23	0.03	0.17	0.09-0.25	<0.001
Distal tubular							
• H-FABP	1	0.10	-0.02-0.23	0.11	-	-	-
	2	0.01	-0.11-0.12	0.9	0.23	0.15-0.31	<0.001
Inflammatory							
• MIF	1	0.02	-0.13-0.17	0.8	-	-	-
	2	-0.07	-0.20-0.06	0.3	0.22	0.14-0.30	<0.001
• MCP-1	1	0.40	0.27-0.52	<0.001	-	-	-
	2	0.28	0.14-0.42	<0.001	0.12	0.04-0.20	0.005

Beta's, confidence intervals and p-values were calculated using multivariable linear regression. Dependent variable is log base 2 transformed TRV, independent variables are the log base2 transformed 24h excretions of the various urinary biomarkers.
Model 1: adjusted for age and gender
Model 2: adjusted for age, gender and albuminuria.
Abbreviations are: IgG, immunoglobulin G; β2 MG, β2 microglobulin; KIM-1, Kidney Injury Molecule 1; NAG, N-acetyl-β-D-glucosaminidase; NGAL, Neutrophil Gelatinase-Associated Lipocalin; H-FABP, Heart-type Fatty Acid Binding Protein; MIF, macrophage migration inhibitory factor; MCP-1, monocyte chemotactic protein-1.





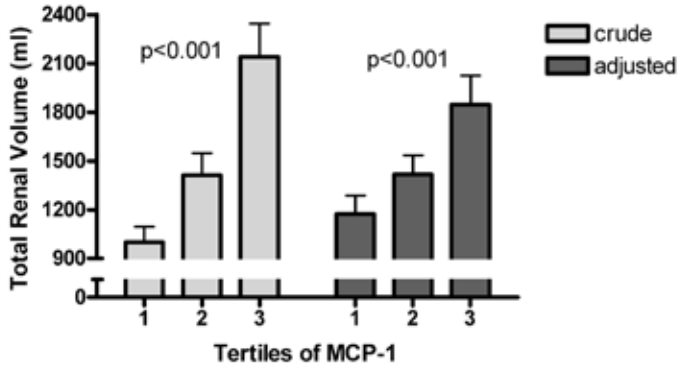


Figure 8: The associations between KIM-1 (upper panel), NGAL (middle panel), and MCP-1 (lower panel), and total renal volume are independent of albuminuria. Tertiles of the biomarkers are shown crude (light grey) and after adjustment of age, gender and albuminuria (dark grey).

Sensitivity analyses

The associations between urinary biomarker excretions with functional and structural measures in ADPKD were essentially the same as the associations between urinary biomarker/creatinine ratios and these functional and structural measures. When all analyses were repeated including only ADPKD subjects with a mGFR >60 mL/min (n=79, not performed with mGFR as a reference test), the results of the crude associations (Table 3) remained the same, except for the association between H-FABP and ERBF, that now did not reach significance when only subjects with an mGFR > 60 mL/min were included.

Discussion

The most important finding of this study is that all urinary biomarkers, be it for glomerular, proximal tubular, distal tubular damage or for inflammation, were elevated in ADPKD patients, when compared to healthy controls. Furthermore, NGAL was associated with renal blood flow and total renal volume, independent of albuminuria and is therefore an interesting candidate marker to predict disease progression. In addition to that, β2 microglobulin and H-FABP were inversely associated with glomerular filtration rate and effective renal blood flow, independent of albuminuria and KIM-1, NGAL and MCP-1 were positively associated with total renal volume, independent of albuminuria. Albuminuria correlated well with glomerular filtration rate, effective renal blood flow and total renal volume.

We found all urinary biomarkers to be elevated in ADPKD. According to our study, both glomerular, proximal- and distal tubular and the inflammatory marker MCP-1 were elevated in ADPKD compared to healthy controls, suggesting the disease (indirectly) affects multiple parts of the nephron. Glomerular markers were more markedly elevated in the ADPKD patients than tubular damage markers. When patients are studied with a relatively well preserved eGFR (>60 ml/min per 1.73 m2), a relatively well preserved renal blood flow (>500 ml/min) or a relatively modest total renal volume (<1000 ml) all urinary biomarkers (except H-FABP and MIF) were also increased, indicating that the increase in urinary biomarkers occurs early in the disease process. That H-FABP excretion is elevated only later in the disease process is in contrast with literature that suggests that the main site of cystogenesis is distal in the tubules[39]. Of note, we found a striking correlation between IgG and albuminuria, which could suggest albuminuria in ADPKD is predominantly glomerular in origin. Furthermore, biomarkers were associated with either glomerular filtration rate and renal blood flow or total renal volume. Also in the ROC curves, the biomarkers with highest area under the curve are different for TRV and ERBF/mGFR. NGAL was the only urinary biomarker that remained associated with mGFR, effective renal blood flow and with total renal volume.

An increased urinary albumin excretion is a risk factor for ADPKD disease progression (renal growth and renal function deterioration).[9] Apart from albuminuria however, the studies that have been published on urinary biomarkers in ADPKD, are nearly always performed in small sample size populations and not corroborated by others. Urinary β2 microglobulin was elevated in 14 ADPKD patients compared to 6 healthy controls.[15] KIM-1 was expressed in murine polycystic kidneys, but not in normal kidneys.[40] To our knowledge however, urinary KIM-1 in human ADPKD has never been measured, nor are we aware of any studies describing urinary

NAG levels in ADPKD. For NGAL, serum and urinary levels were higher in 26 ADPKD patients compared to 26 healthy volunteers.[16] Urinary fatty acid binding protein excretion is previously described to be elevated in ADPKD.41 However, in this study liver-fatty acid binding protein was measured and not heart fatty acid binding protein, as in our study. Increased levels of MCP-1 in cyst mural cells and in urine was associated with monocyte accumulation within the renal interstitium in a rat model of ADPKD.[42] Also, in ADPKD patients, an increased urinary excretion of MCP-1 has been described.[17;18] MCP-1 is thought to play a critical role in the responses of macrophage migration inhibition factor (MIF). No studies so far have described urinary MIF excretion in ADPKD as yet. In one study, in polycystic kidney disease, multiple urinary biomarker tests were compared, namely albuminuria and urinary β-N-acetlyhexosaminidase and its isoenzymes.[43]

The question arises whether the markers we measured are only markers of disease activity, or whether they are also involved in the pathophysiologic process of cyst formation. Experimental studies suggest that KIM-1 is probably involved in phagocytosis.[44] It is also an endogenous ciliary protein[45] and could be involved in regulating flow-induced calcium signalling.[46] NGAL suppressed cyst growth by PKD1 null cells in vitro and in mice.[47] Because of the observational nature of our study, we can only speculate whether the markers we found are indeed involved in the pathophysiology of ADPKD.

We acknowledge that our study has limitations. First, our study is cross-sectional, which could induce selection bias and makes that we are not able to look at associations between the markers and prediction of disease progression. Future studies will have to establish the clinical utility of these urinary biomarkers in predicting prognosis or response to therapy. Second, being a single centre study, our study needs of course external validation. Third, we do not know whether the found elevation of urinary biomarkers is specific for ADPKD or could also occur in non-ADPKD patients with chronic kidney disease. The increased urinary biomarker excretion could be caused by non-specific injury rather than the cystogenic process. Of note, we investigated whether urinary biomarkers can improve the assessment of disease severity in clinical practice. Therefore we did not take serum levels into account. Assessment of albuminuria is often performed in clinical practice, we attempted to identify urinary biomarkers that are of additional value to albuminuria. Strengths of our study are that we measured multiple markers in a relatively large cohort, consisting of 102 ADPKD patients, at different stages of the disease, and associated these biomarkers with gold standard measurements of renal blood flow and total renal volume, enabling a comparison between these biomarkers.

In summary, we found urinary biomarkers from all segments of the nephron to be elevated in ADPKD patients compared to healthy controls. NGAL was associated with both renal blood flow, as well as total with renal volume, independent of albuminuria. We found associations that were independent of albuminuria between β2 microglubulin and H-FABP versus effective renal blood flow and of KIM-1, NGAL and MCP-1 and total renal volume. Based on these cross-sectional data, we hypothesize that determination of β2 microglobulin, KIM-1, H-FABP, MCP-1, and especially NGAL could have additional value in clinical practice to assess disease severity in ADPKD.

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Part II
Chapter 4

Copeptin, a surrogate marker of vasopressin, is associated with micro-albuminuria in a large population cohort.

Kidney Int 77:29-36, 2010

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Abstract
Urinary albumin excretion is a powerful predictor of progressive cardiovascular and renal disease. In rats and humans, administration of a synthetic vasopressin analogue, 1-desamino-8-D-vasopressin, increases urinary albumin excretion; however, it is unknown if endogenous vasopressin levels influence albumin excretion.
To determine this, we measured copeptin, a marker of endogenous vasopressin levels, and its association with urinary albumin excretion in 7,593 subjects who participated in the PREVEND study, a prospective population based, observational cohort. Urinary albumin excretion was measured in two consecutive 24h urine samples by nephelometry while copeptin was measured by an immuno-assay.
Median copeptin concentrations were significantly higher in males than females and high levels were associated with significantly lower 24h urine volumes of high osmolarity. With increasing quintiles of copeptin levels, the percentage of microalbuminuric subjects increased from 13 to 25 for males and from 8 to15 for females. This association was independent of age and other potential confounders; however, we found an interaction between age and copeptin in their association with urinary albumin excretion.
Our study shows that plasma copeptin levels are associated with microalbuminuria, consistent with the hypothesis that vasopressin is involved in causing urinary albumin excretion. If future studies show that this relation is causal, then drinking more water or pharmacological intervention to decrease plasma vasopressin may have beneficial effects on the kidney, especially in the elderly.

Introduction
Urinary albumin excretion (UAE) is a powerful predictor of cardiovascular mortality and progressive renal function deterioration, not only in diabetics, but also in the general population.[1;2] It is assumed that UAE not only reflects glomerular damage, but is also related to systemic endothelial dysfunction. Lowering albuminuria is associated with a better renal[3] and cardiovascular[4] outcome. Identifying modifiable factors that cause a rise in UAE is, therefore, important, since intervention directed to these factors might be expected to result in a better renal and cardiovascular prognosis.
Vasopressin has been hypothesized to be one of these modifiable factors. Vasopressin is also known as antidiuretic hormone since it binds to the V2 receptors in the collecting ducts, inducing the insertion of the molecular water channel aquaporin-2 in the luminal membrane of principal cells. This mediates water reabsorption, thereby reducing water excretion[5]. Despite its importance for normal water regulation in the body, vasopressin also has been reported to exert deleterious effects on the kidney. Administration of 1-desamino-8-D-arginine vasopressin (a vasopressin receptor agonist) to rats and humans induces albuminuria[6;7] and inhibition of vasopressin by drinking water or V2 antagonism, reduces proteinuria in rats with renal failure[8;9] and diabetes.[10]
Interest in pleiotropic effects of vasopressin has risen because vasopressin antagonists are expected to become available for clinical use within the next years. Human studies investigating the influences of endogenous vasopressin on albuminuria are, however, lacking. Presumably this is caused by the fact that direct measurement of vasopressin in humans is problematic. More than 90% of vasopressin in the circulation is bound to platelets,[11] vasopressin is unstable in isolated plasma11 and most vasopressin assays have relatively limited sensitivity. Recently, an assay has been developed to measure copeptin, the C-terminal portion of the precursor of vasopressin. Copeptin has been proven to be a reliable marker of vasopressin secretion and a useful substitute for circulating vasopressin concentration in clinical routine.[12;13] These characteristics make copeptin a promising marker, which allows for the first time (indirect) measurement of vasopressin in epidemiological studies.
Given the aforementioned, we hypothesized that in the general population copeptin (a surrogate marker of vasopressin) is associated with albuminuria, and investigated this in an observational, cross-sectional study.

Materials and Methods
Study Design and Population
This study is part of the Prevention of Renal and Vascular End Stage Disease (PREVEND) Study, a cohort study that investigates the predictive value of UAE for renal and cardiovascular disease progression. All inhabitants of the city of Groningen between the ages 28 and 75 years (85,421 subjects) were asked to send in a morning urine sample and to fill a short questionnaire on demographics and cardiovascular history. A total of 40,856 subjects (47.8%) responded. After exclusion of insulin dependent diabetes mellitus and pregnant women, all others with urinary albumin concentration ≥ 10 mg /l, together with a randomly selected control group, were invited. Finally, 8592 subjects completed the total screening program, rendering the actual

screening cohort. Details of this study have been published elsewhere.[14;15] For this study, subjects with a missing copeptin value (n=888, missing samples), missing 24h urine collections (n=8) or non-fasting blood sample (n=103) were excluded, leaving 7593 subjects for analysis. The PREVEND study is approved by the medical ethics committee of our institution and was conducted in accordance with the guidelines of the Declaration of Helsinki. All participants gave written informed consent.

Measurements and definitions

Participants completed two visits at our outpatient unit. Height and weight were measured. Information on drug use was given by data from community pharmacies. During the first and the second visit, blood pressure was measured on the right arm, in supine position, every minute for 10 and 8 minutes, respectively, with an automatic device (Dinamap XL Model 9300; Johnson-Johnson Medical, Tampa, FL). Two 24h urine samples were collected after thorough oral and written instructions on how to perform urine collection, and fasting blood sample was drawn.

Urinary albumin concentration was determined by nephelometry (BNII; Dade Behring Diagnostics, Marburg, Germany). Concentrations of sodium and potassium were measured in urine, and cholesterol and glucose levels were measured in serum using standard methods. Creatinine concentration was measured using Kodak Ektachem dry chemistry (Eastman Kodak, Rochester, NY, USA). Copeptin was measured using a new sandwich immunoassay (B.R.A.H.M.S. AG, Hennigsdorf / Berlin, Germany), which was based on the assay described previously.[12]

The assay was modified as follows: the capture antibody was replaced by a murine monoclonal antibody directed to amino acids 137-144 of proAVP. This modification improved the sensitivity of the assay. The lower detection limit was 0.4 pmol/L and the functional assay sensitivity (20% interassay coefficient of variation) was less than 1 pmol.[16]

BMI was calculated as weight in kilograms (kg) divided by height in square meters (measured to the nearest 0.5 kg and 0.5 cm, respectively). History of coronary heart disease was defined as self-reported myocardial infarctions, cardiac operations, percutaneous transluminal coronary angioplasty or cerebrovascular accident. This definition of coronary heart disease would probably misclassify subjects with unrecognized or silent coronary heart disease.[17]

Systolic and diastolic blood pressure were measured as mean of the last two measurements of the two visits. These blood pressure values were used to calculate a mean arterial pressure using the standard formula 2/3 diastolic blood pressure + 1/3 systolic blood pressure. UAE was calculated as the mean of the two 24h urine excretions. Included in our definition of diuretic use are all agents mentioned under C03 of the Anatomical Therapeutic Chemical (ATC) classification system (low-ceiling diuretics, thiazides, low-ceiling diuretics, excl thiazides, high-ceiling diuretics, potassium-sparing agents, diuretics and potassium sparing agents in combination and combinations including these agents). Diabetes was defined as use of oral antidiabetic medication, or a fasting glucose >7.0 mmol/l. Sodium excretion was used as a surrogate for sodium intake. Creatinine values were used to calculate an eGFR, using the modification of diet in renal disease formula.[18] In early PREVEND papres, creatinine has not been reported as IDMS-traceable creatinine. To keep reporting of creatinine in PREVEND manuscripts consistent over

time, creatinine is always provided as non-IDMS traceable values. We have, however, validated creatinine against IDMS and used this as an additional sensitivity analysis. Microalbuminuria was defined as an UAE > 30 mg/24h. Urinary osmolarity was calculated as 2 * (Urinary sodium concentration +Urinary potassium concentration) + Urinary urea concentration. This formula is not validated in our cohort, but extracted from literature.[19-21]

Statistical Analyses

Analyses were performed with SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Parametric variables are expressed as mean ± s.d., whereas non-parametric variables are given as median (interquartile range). Skewed data, such as C-reactive protein (CRP), were normalized by logarithmic transformation in analyses. A two sided $p < 0.05$ was considered to indicate statistical significance.

To visualize associations with copeptin, the study population is subdivided into quintiles of copeptin concentration. Since males have a significantly higher copeptin concentration than females,13 when depicted not specifically separately, these quintiles were stratified for gender. P-values for differences between these quintiles were obtained using ANOVA or Kruskal-Wallis test.

Gender-stratified quintiles were also made for BMI, blood pressure, cholesterol, fasting glucose and CRP (other modifiable factors that are known to be associated with an increase in UAE in the non-diabetic population[22]) . This was done to compare the association between copeptin concentration and UAE with the association between these other modifiable factors and UAE.

To investigate whether copeptin concentration is associated with other variables, multiple regression analysis was performed. Logarithmic transformation of copeptin was applied to fulfill the requirement of equal distribution of the residuals. This was performed for the whole group and for males and females separately, both crude and adjusted for age.

To investigate the association between copeptin and UAE, logarithmic transformation of UAE was applied and linear regression was performed. Stepwise, various models were built to adjust for possible confounding variables for males and females separately. First, the association between log copeptin and log UAE was investigated crude; second, it was adjusted for age and gender; third, adjusted for potential cardiovascular confounders (MAP, BMI, smoking, glucose, cholesterol, CRP, eGFR); and, finally, adjusted for medication that could potentially influence both vasopressin secretion as well as UAE (diuretics and ACEi/ARB). The same models were used to investigate the association between urinary osmolarity and UAE in multiple regression analyses. Interactions between baseline characteristics and log copeptin were tested in the multivariate model with UAE being the dependent variable.

Various sensitivity analyses were performed. To test whether use of diuretics would not only influence copeptin concentration, but also influence UAE, the analyses were also performed separately for subjects with and without diuretic use. Analyses were also performed stratified for medication influencing the renin-angiotensin system (ACEi and ARB's, yes/no) and for diabetic status (yes/no). All analyses were repeated, first, without outliers for copeptin (defined as a measurement which falls more than 1.5 times the interquartile range above the third quartile or below the first quartile;[23] second, we repeated the analyses with creatinine values that were

IDMS traceable. Finally, all analyses were repeated excluding subjects with >20% difference in creatinine excretion in two consecutive 24h urine collections as this may indicate incompleteness of urine collection.

Results

Characteristics of participating subjects are presented for quintiles of copeptin in Table 1 separately for gender. A total of 7,593 subjects (47.2% male, aged 49 ±13 years) were analyzed. Median copeptin concentration in these subjects was 4.7 (2.9-7.5) pmol/l, with significantly higher concentration in males (6.2 (4.1-9.5) pmol/l) than in females (3.6 (2.4-5.5) pmol/l, $p < 0.001$). Table 2 depicts associations between copeptin and these variables for males and females. As copeptin concentration is significantly associated with age, an additional column is included to represent the association adjusted for age. When adjusted for age, copeptin concentration was positively associated with serum creatinine, blood pressure, body mass index (BMI) and sodium intake in males and females, whereas the association with eGFR was negative. Associations between copeptin and smoking and use of angiotensin-converting enzyme inhibitors/ angiotensin-II-recepor blockers (ACEi/ARBs) are significant in females and not in males, while plasma glucose and plasma cholesterol levels are significant in males but not in females. The association between copeptin and blood pressure is presented in the table for the whole group. For subjects on antihypertensive medication (males n=494, females n=510) this association is not significant. For those who do not receive antihypertensive treatment, the associations between copeptin and blood pressure are approximately similar to that of the whole group (depicted in Table 2).

Table 1: Characteristics of participating subjects (N = 7,593) per quintile of copeptin for males (M) and females (F).

Quintile		*1*	*2*	*3*	*4*	*5*
copeptin (pmol/l)	M	0.6-3.7	3.8-5.3	5.4-7.3	7.4-10.4	10.5-632
	F	0.1-2.1	2.2-3.0	3.1-4.2	4.3-6.2	6.3-131
n	M	730	726	696	716	715
	F	772	829	809	803	797
Age (y)	M	49 ± 13	48 ± 13	50 ± 13	49 ± 12	52 ± 13
	F	47 ± 12	47 ± 12	48 ± 12	48 ± 12	50 ± 12
History of CHD, n (%)	M	58 (8)	43 (6)	55 (8)	63 (9)	69 (10)
	F	21 (3)	15 (2)	20 (3)	21 (3)	25 (3)
Smoking, n (%)	M	113 (15)	123 (17)	118 (17)	126 (18)	95 (13)
	F	98 (13)	94 (11)	136 (17)	157 (20)	165 (21)
BMI, kg/m^2	M	25.7 ± 3.3	25.9 ± 3.3	26.4 ± 3.6	26.5 ± 3.7	26.7 ± 4.0
	F	25.4 ± 4.1	25.6 ± 4.5	25.8 ± 4.7	26.3 ± 4.9	26.2 ± 5.4
MAP, mm Hg	M	93.4 ± 11.3	94.3 ± 10.5	95.2 ± 11.6	95.6 ± 11.1	98.5 ± 12.3
	F	87.9 ± 11.8	88.5 ± 11.8	88.2 ± 11.4	88.9 ± 12.4	90.0 ± 12.6
Use of diuretics, n (%)	M	19 (3)	21 (3)	21 (3)	20 (3)	39 (5)
	F	40 (5)	39 (5)	28 (3)	42 (5)	48 (6)
Use of ACEi / ARB, n (%)	M	42 (6)	35 (5)	34 (5)	33 (5)	52 (7)
	F	27 (3)	28 (3)	22 (3)	34 (4)	44 (6)
Sodium intake, mmol/day	M	154 ± 51	161 ± 53	163 ± 54	163 ± 57	156 ± 54
	F	123 ± 42	126 ± 41	127 ± 42	125 ± 42	127 ± 47
Plasma glucose, mmol/l	M	4.9 ± 0.9	4.9 ± 0.9	4.9 ± 0.9	5.1 ± 1.3	5.3 ± 1.8
	F	4.7 ± 0.8	4.8 ± 1.3	4.7 ± 0.9	4.7 ± 0.9	4.8 ± 1.3
Diabetes, n (%)	M	26 (4)	26 (4)	18 (3)	26 (4)	50 (7)
	F	18 (2)	28 (4)	23 (3)	18 (2)	28 (4)
Plasma cholesterol, mmol/l	M	5.6 ± 1.1	5.6 ± 1.1	5.6 ± 1.1	5.7 ± 1.1	5.8 ± 1.2
	F	5.6 ± 1.2	5.5 ± 1.1	5.6 ± 1.2	5.6 ± 1.1	5.8 ± 1.2
Serum CRP, mg/l	M	1.0 (0.5-2.3)	1.0 (0.5-2.4)	1.2 (0.6-2.7)	1.3 (0.6-3.0)	1.4 (0.7-3.0)
	F	1.2 (0.5-3.2)	1.2 (0.5-3.0)	1.4 (0.6-3.2)	1.4 (0.6-3.3)	1.5 (0.6-3.6)
Serum creatinine, umol/l	M	89 ± 11	90 ± 13	91 ± 13	92 ± 13	98 ± 41
	F	74 ± 9	75 ± 10	76 ± 10	77 ± 10	78 ± 17
eGFR, ml/min*1.73m^2	M	87 ± 14	86 ± 15	84 ± 14	83 ± 14	80 ± 16
	F	80 ± 13	79 ± 13	78 ± 13	77 ± 13	75 ± 15

Parametric variables are expressed as mean ± SD, whereas non-parametric variables are given as median (interquartile range).

Table 2: associations between copeptin and other variables for males and females

variable	*males*				*females*			
	crude		age adjusted		crude		age adjusted	
	St. β	P	St. β	P	St.β	P	St. β	P
Age	0.07	<0.001	-	-	0.09	<0.001	-	-
History of CHD	0.02	0.32	-0.01	0.79	0.02	0.19	0.01	0.67
Smoking	-0.02	0.34	-0.01	0.51	0.12	<0.001	0.13	<0.001
BMI	0.09	<0.001	0.07	<0.001	0.06	<0.001	0.04	0.03
MAP	0.15	<0.001	0.15	<0.001	0.05	<0.001	0.01	0.48
Use of diuretics	0.05	0.002	0.04	0.03	0.04	0.021	0.02	0.32
Use of ACEi / ARB	0.02	0.24	0.03	0.07	0.05	0.001	0.05	0.002
Sodium intake	0.03	0.12	0.03	0.041	0.03	0.10	0.04	0.019
Plasma glucose	0.10	<0.001	0.0	<0.001	0.03	0.03	0.01	0.69
Diabetes	0.07	0.001	0.05	0.006	0.02	0.24	0.00	0.85
Plasma cholesterol	0.07	<0.001	0.06	<0.001	0.06	<0.001	0.03	0.14
Serum CRP	0.05	0.002	0.04	0.015	0.04	0.03	0.03	0.06
Serum creatinine	0.18	<0.001	0.18	<0.001	0.18	<0.001	0.17	<0.001
eGFR	-0.17	<0.001	-0.18	<0.001	-0.15	<0.001	-0.13	<0.001

To investigate whether vasopressin concentration is consistent with normal physiology, we tested the associations between copeptin and 24h urinary volume and 24h urinary osmolarity. A high concentration of copeptin was associated with a low 24h urinary volume (in males R= -0.27 and in females R = -0.23, both p <0.001) and high urinary osmolarity (in males R=0.27 and in females R=0.23, both p<0.001). The correlation of copeptin with overall calculated urinary osmolarity was stronger than with the single determinants of the calculated osmolarity (for copeptin and urinary sodium concentration R-value was 0.25 in males and 0.22 in females; for urinary potassium concentration this was 0.16 for males and 0.15 for females; and for urinary urea concentration, R-value was 0.25 and 0.21, respectively). The associations of copeptin with 24h urinary volume and 24h urinary osmolarity are presented in Figure 1. Twenty-four-hour urinary volume is highest in quintile 1 and lowest in quintile 5 (1.74 ± 0.51 versus 1.36 ± 0.44 L for males and 1.82 ± 0.60 versus 1.43 ± 0.52 L for females) whereas urinary osmolarity varies in an opposite manner (533 ± 155 versus 667 ± 183 mOsm/L for males and 418 ± 133 versus 530 ± 170 mOsm/L for females). The osmolar load is not different between quintile 1 and quintile 5 for each gender (for males 876 mOsmol in quintile 1, versus 841 osmol in quintle 5, p =0.06 and for females 707 versus 698 mOsmol, p=0.3).

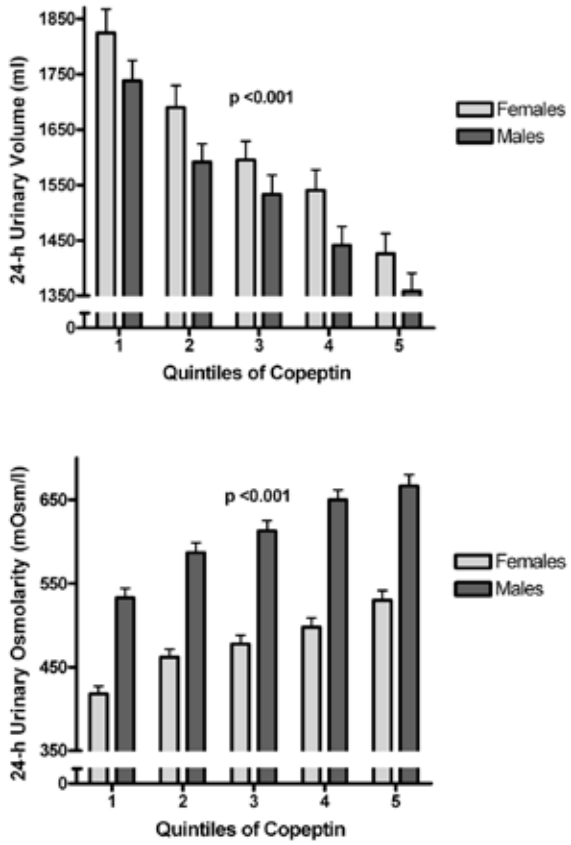

Figure 1: Associations between quintiles of copeptin and 24h urinary volume (upper panel) and 24h urinary osmolarity (lower panel) for males and females. Depicted are mean values (± 95% confidence interval of the mean). Differences between the quintiles were tested by analysis of variance.

Median UAE for the whole group was 9.3 (6.4-16.8) mg per 24 hour and 986 (13%) of the subjects had micro-albuminuria (micro-albuminuria is defined as a UAE ≥30 mg per 24h). Copeptin level was significantly associated with UAE (R = 0.20, p <0.001). This association is depicted in Figure 2. UAE is higher in the quintile with higher copeptin values: in quintile 1: 9.4 (6.5-17.2) mg/24h for males and 7.3 (5.5-11.7) mg/24h for females and in quintile 5: 13.4 (8.1-29.8) mg/24h for males and 10.0 (6.6-18.3) for females. Also depicted in Figure 2 is the percentage of microalbuminuric subjects per quintile of copeptin. The percentage of microalbuminuric subjects is highest in the highest quintile of copeptin.

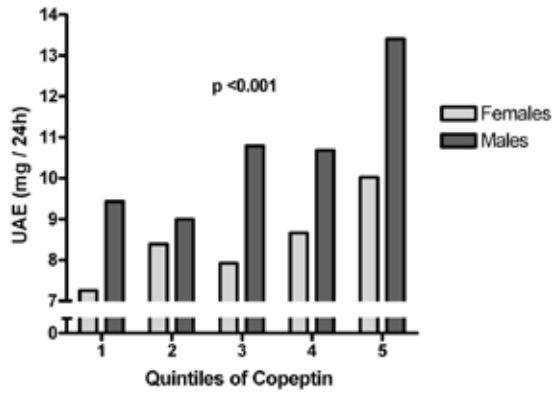

Figure 2: Association between quintiles of copeptin and median 24h urinary albumin excretion (UAE, upper panel) and prevalence of micro-albuminuria (lower panel) for males and females. Differences between the quintiles were tested by Kruskal Wallis test.

In Figure 3, the association between copeptin and UAE is shown in addition to that of modifiable factors that are known to influence UAE (mean arterial blood pressure (MAP), BMI, fasting plasma glucose and cholesterol). For this purpose gender-stratified quintiles were made for every factor specifically. This figure shows that the association between MAP and UAE is the strongest and that the association between copeptin and UAE is comparable to that between glucose, BMI or cholesterol and UAE. R-values for the association between MAP, glucose, BMI and cholesterol versus UAE are respectively 0.36, 0.25, 0.21 and 0.12.

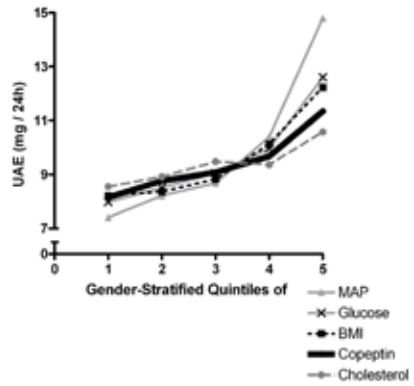

Figure 3: Association between copeptin and UAE (upper panel) and copeptin and micro-albuminuria (lower panel) compared with known modifiable factors influencing UAE. Depicted are gender-stratified quintiles of copeptin concentration / Mean Arterial Pressure (MAP) / body mass index (BMI) / fasting plasma glucose and plasma cholesterol. Copeptin quintiles are depicted in black (thick line).

The association between copeptin and UAE was also investigated using copeptin as a continuous variable in multivariate regression analyses. We adjusted this association in a multivariate model for several potential confounders. Despite the fact that our regression model did not show indications for an interaction with gender and that the regression coefficients for males and females were not different (p=0.11 for the crude model and 0.37 for the fully adjusted model), results are depicted for males and females separately in Table 3. The association was significant with a beta-value of 0.25 for males and 0.19 for females, both p < 0.001 (Table 3, model 1). Adjusted for gender, the association remained significant. Further adjustment for potential cardiovascular confounders (model 3), and for medication that could potentially influence both vasopressin (and thus copeptin concentration) and UAE (model 4), did not materially influence the association between copeptin and UAE.
Urinary osmolarity was also associated with UAE with a standardized beta-value of -0.05 for males and 0.07 for females, both p <0.001. This association was independent of age, gender, potential cardiovascular confounders and use of diuretics and/or ACEis/ARBs.

Table 3 Associations between log copeptin concentration and log UAE in univariate (model 1) and multivariate models. Log copeptin concentration was entered in the regression analyses as independent variable, log UAE as the dependent.

males

model	*corrected for*	*β*	*95% CI for β*	*P*
1	- (crude)	0.25	0.20-0.30	<0.001
2	As 1 + age	0.21	0.16-0.26	<0.001
3	As 2 + MAP, BMI, smoking, glucose, cholesterol, CRP and eGFR	0.10	0.05-0.16	<0.001
4	As 3 + diuretics and ACEi/ARB.	0.09	0.04-0.15	0.001

females

model	*corrected for*	*β*	*95% CI for β*	*P*
1	- (crude)	0.19	0.15-0.23	<0.001
2	As 1 + age	0.17	0.14-0.22	<0.001
3	As 2 + MAP, BMI, smoking, glucose, cholesterol, CRP and eGFR	0.16	0.11-0.21	<0.001
4	As 3 + diuretics and ACEi/ARB.	0.17	0.12-0.21	<0.001

In the crude and the full corrected model, an interaction was found between age and log copeptin on UAE ($p < 0.001$). In Figure 4, the association between copeptin and UAE is depicted stratified for quintiles of age. Copeptin was associated with UAE in all age groups, but this association is the strongest when subjects are older. In the youngest age quintile (age 28-36y), copeptin concentration rose from 1.9 (1.6-2.7) to 11.2 (8.3-14.4) pmol/l from the first to the fifth quintile of copeptin, with median UAE rising from 7.5 (5.8-10.4) to 8.5 (6.2-13.6) mg /24h. In the oldest age quintile of our study (age 62-75y), copeptin concentration rose from 2.0 (1.5-2.8) to 11.9 (9.3-15.8) pmol/l while median UAE rose from 11.1 (7.0-23.5) to 18.2 (9.4-40.8) mg/24h. Twenty-four-hour urinary volume and 24h urinary osmolarity were significantly different, with 24h urinary volume being higher and 24h urinary osmolarity being lower in the oldest age group when compared to the youngest age group (in the highest quintile of copeptin, 1434 ± 465 ml and 543 ± 163 mOsm/l for the oldest quintile versus1259 ± 485 ml and 673 ± 202 mOsm/l in the youngest quintile).

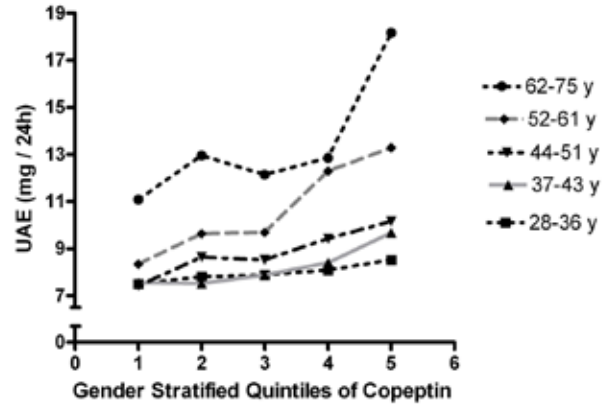

Figure 4: Interaction between age and copeptin in the association with urinary albumin excretion (UAE). Depicted are median UAE values according to gender-stratified quintiles of copeptin for the various age groups.

Of note, apart from the interaction of age and log copeptin on UAE, we did not find any other interactions between baseline characteristics and log copeptin on UAE. The results we obtained in the various sensitivity analyses (stratified for ACEi/ARB and diuretics use (yes / no), with or without outliers of copeptin, with or without diabetic subjects and without subjects with a non-fasting blood sample and without subjects with >20% difference in creatinine excretion in two consecutive 24h urine collections and repeated with IDMS traceable creatinine values), were essentially similar to those from our primary analyses.

Discussion

In the present study, we found copeptin values to be higher in males than in females, corresponding with lower urine volumes and higher urinary osmolarity in males versus females. In both males and females, high copeptin concentration (a surrogate for vasopressin) is associated with low 24h urinary volume and high 24h urinary osmolarity. Furthermore, our study shows that copeptin is associated with UAE: the higher copeptin, the higher UAE and the more microalbuminuric subjects, again, both in males and in females. This association remained significant after adjustment for age, potential cardiovascular confounders and medication that could potentially influence both vasopressin (and thus copeptin) secretion and UAE. The association between copeptin and UAE was found to be most pronounced for older subjects. The observed association between vasopressin and UAE is consistent with our hypothesis that vasopressin induces albuminuria. Animal studies showed that administration of 1-desamino-8-D-arginine-vasopressin (a V2-receptor agonist) induced an increase in urinary protein excretion and a more rapid decline of renal function.[24] Also in humans, short-term 1-desamino-8-D-arginine-vasopressin administration has been shown to result in an increase in UAE. 6 In contrast, vasopressin inhibition by drinking water8 or by V2 antagonism[25] led to a decrease in proteinuria in rats with renal failure.

In a rat model of diabetes mellitus, chronic treatment with a vasopressin V2 antagonist prevented the rise in albuminuria seen in untreated rats.10 In these untreated rats with diabetes mellitus, albuminuria was significantly correlated with free-water reabsorption, suggesting an association between albuminuria and vasopressin.
The unfavorable renal effects of vasopressin have been argued to be partly due to an effect on blood pressure. Vasopressin may contribute to hypertension either by a direct effect on vascular smooth muscle through activation of the V1a receptor,[26] or by V2 receptor-dependent tubular effects. The latter includes an enhancement of sodium reabsorption in the collecting duct,[27] and an increase in intrarenal urea recycling which participates in the urine-concentrating mechanism. The latter may, indirectly modify NaCl concentration at the macula densa, thereby influencing tubuloglomerular feedback, leading to renin release, hypertension, glomerular hyperfiltration, and proteinuria.[6;28] In our study however, the association between copeptin and UAE was independent of systemic blood pressure, indicating that other mechanisms of action may be important. Furthermore, urinary osmolarity was independently associated with UAE, but less strong that the association between copeptin and UAE, suggesting that besides the antidiuretic effect of vasopressin, also other effects (e.g. pressor effects) might be involved in the relationship of copeptin with UAE. Induction of specific glomerular hyperfiltration or decreased tubular albumin reabsorption might be mechanisms underlying this relationship.
We also found copeptin to be associated with renal function. Subjects with higher levels of copeptin had lower renal function. Reasons for this phenomenon could be that copeptin causes renal function decline or that subjects with low renal function are less sensitive to the actions of copeptin / vasopressin. The fact that copeptin is partly cleared by the kidney19 is not likely to be an explanation, because negative feedback loops will downregulate copeptin/ vasopressin secretion.
We investigated potential interactions between log copeptin on the one hand and gender, age and baseline renal function on the other hand, because we wanted to investigate whether there are certain subgroups of subjects that are more susceptible to copeptin's influences. We found no interaction between copeptin and gender or renal function, suggesting that the association between log copeptin and UAE is essentially similar in men and women and independent of renal function. The interaction between age and copeptin was, however, significant, suggesting that the association between log copeptin and UAE is most pronounced in older subjects (Figure 4). This could be explained because they might be more vulnerable to the influence of vasopressin or because they have been longer exposed to high vasopressin levels. Another explanation for this interaction could be that older subjects might have decreased tubular reabsorption. This could result in both an increased UAE and decreased concentration capacity (24h urinary volume is higher and 24h urinary osmolarity is also lower in the oldest quintile) or become resistant to vasopressin with age.

The association between copeptin and UAE has an R-value of 0.20. This relatively low R-value indicates that other factors also contribute to UAE. Compared with well acknowledged modifiable risk factors for UAE, the association between copeptin and UAE is less strong than between MAP and UAE, but of approximately similar strength as BMI, cholesterol and glucose (indicated

by Figure 3 and the R-values of these associations). It is furthermore important to note that the association between copeptin and UAE was independent of these potential confounders (Table 2).

Copeptin levels are likely to fluctuate day by day according to changes in volume or plasma osmolarity. This questions whether the found association will be of relevance for renal function or albuminuria in the long run. There is, however, evidence that copeptin levels are not only dependent on volume status and plasma osmolarity, but also to a certain extent are genetically determined. Subjects who have higher thirst and vasopressin threshold as genetic trait,[29-31] could, therefore, be at greater renal risk. These subjects with higher vasopressin level as a genetic trait and / or elderly subjects are most likely to profit from intervention (as drinking water or administration of a vasopressin-receptor antagonist).

We acknowledge that this study has limitations. First, it is a cross-sectional epidemiologic study. Therefore no certain causal relationship can be proven. A third factor could underlie both copeptin level and albuminuria. For instance, subjects with higher UAE have more risk factors and a greater extent of subclinical interstitial injury and subsequent reduction of urine-concentration capacity. The observation that the association remains significant in the multivariate regression analysis suggests that this association is independent of possible confounding factors. It is, however, possible that residual confounding remained even in multivariate-analysis, because it is hard to adjust completely for the duration or severity of each risk factor, for extent of tubulo-interstitial injury and impairment of water-concentration capacity. Additional research is needed to point out a mechanism underlying the observed association. Furthermore, our study consisted of predominantly Caucasian people, making extrapolation of our results to other ethnicities difficult.

Strengths of our study, as far as we know, are that, this is the first study to investigate the association between endogenous vasopressin and UAE. It confirms the results obtained with 1-desamino-8-D-arginine-vasopressin administration in animals and humans. Furthermore, this study has been performed in a large number of subjects with extensive information on a large number of covariates.
In conclusion, our study shows that copeptin (a reliable substitute for vasopressin) is associated with UAE and microalbuminuria, consistent with the hypothesis that vasopressin induces UAE. If it is true that vasopressin induces albuminuria, suppressing vasopressin by administration of a vasopressin-receptor antagonist or simply by drinking more water might be beneficial for the kidney, especially in the elderly.

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Part II
Chapter 4.1

Vasopressin and microalbuminuria: is it vasopressin per se or is it salt intake?

Kidney Int 77:832-833, 2010

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To the Editor:

In his commentary,[1] dr Cirillo focuses on the Prevention of Renal and Vascular Endstage Disease (study) data showing that copeptin plasma levels are associated with albuminuria.[2] He emphasizes that vasopressin needs to be paid more attention when analyzing kidney dysfunction. He also raises two questions, which we would like to answer.

First, he points out that the inverse association between vasopressin and glomerular filtration rate (GFR) that we found22 seems in contradiction to the increase in GFR that Bankir and colleagues reported after vasopressin administration in short-term intervention studies.[3] A possible explanation could well be that exposure to vasopressin in the short-term induces glomerular hyperfiltration, and that this hyperfiltration will in the long-term be detrimental. Indeed, Bankir et al showed in an animal model that long-term administration of a vasopressin V2 receptor agonist caused an increase in proteinuria and a more rapid decline in renal function.[4] In analogy, we described recently that higher baseline endogenous vasopressin levels were associated with a more rapid decline in renal function decline during follow-up in a cohort of renal transplant recipients.[5] In our opinion, our data and those of Bankir et al are therefore not contradictory, but in line.

Second, dr. Cirillo raises the suspicion that the association between vasopressin and albuminuria might not be due to the effects of vasopressin per se, but may be the result of another variable that determines vasopressin release, such as salt intake. He argues that a high salt intake could induce a higher plasma osmolarity and thus vasopressin release, and that a high sodium intake is also associated with albuminuria, but through another mechanism. Of note, we previously indeed described an association between sodium intake and albuminuria in the Prevention of Renal and Vascular Endstage Disease (study) cohort.[6] In response to this question, we added sodium intake (assessed as 24-h urinary sodium excretion) to our multivariate model. The addition of sodium intake did not change the beta value in males (0.09 (P=0.001) vs 0.09 (p=0.001), respectively) and only marginally changed the value in females (from 0.17 (p<0.001) to 0.15 (p<0.001)). This observation makes it less likely that high salt intake is the common factor that induces an increase in albuminuria as well as in vasopressin, and pleads for the option that vasopressin per se is detrimental.

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Part II

Chapter 5

Copeptin, a surrogate marker of vasopressin, is associated with accelerated renal function decline in renal transplant recipients.

Transplantation 88:561-567, 2009.

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Abstract
Chronically elevated vasopressin (VP) plasma levels have been shown to induce accelerated renal function decline in rats with chronic renal failure. Whether endogenous VP is a renal risk factor in humans has not been investigated yet. We aimed to investigate whether, in renal transplant recipients, VP concentration is associated with change in renal function during follow-up.
In this prospective study, all consecutive patients visiting our kidney transplant outpatient clinic between August 2001 and July 2003 were asked to participate. Serum creatinine was assessed at baseline and at follow-up. Copeptin, the C terminal portion of the precursor of VP was determined at baseline (immunoassay). Univariate and multivariate regression analyses were performed to investigate the association between copeptin and renal function decline.
Overall, 548 patients were included 6.0 (2.8-11.6) years after transplantation (men 54%, age 52 (43-60) years). Median follow-up was 3.2 (2.7-3.7) years. Median copeptin level was 9.1 (5.0-18.6) pmol/L at baseline. Copeptin was significantly associated with change in estimated glomerular filtration rate (eGFR; MDRD) during follow up. When our study population was subdivided according to gender-stratified tertiles of increasing copeptin concentration, mean changes in eGFR during follow-up were -0.03, -0.44 and -1.06 mL/min*1.73m2 per year. In multivariate regression analysis, the association of copeptin at baseline with change in eGFR during follow-up remained significant after adjustment for age, gender, baseline eGFR and known risk factors for renal function decline.
These findings suggest that in renal transplant patients, VP may play a role in renal function decline.

Introduction
The antidiuretic hormone vasopressin (VP) is crucial for water regulation in the body. Its secretion is stimulated in response to an increase in plasma osmolarity or a decrease in blood volume. VP binds to the V2 receptors in the collecting ducts, inducing the insertion of the molecular water channel aquaporin-2 in the luminal membrane of principal cells. This mediates water reabsorption, thereby reducing water excretion.[1,2]
Despite its relevance for normal physiology, several unfavorable effects of VP have been reported on the kidney by affecting renal hemodynamics. Experimental studies have shown that a sustained V2 receptor stimulation results in increased renal plasma flow and particularly in increased Glomerular Filtration Rate.[3] This hyperfiltration may have deleterious consequences, in particular in diseased kidneys, resulting in renal hypertrophy,[4] proteinuria,[5] and accelerated renal function decline.[6-8] This proposed mechanism is supported by studies in rats with chronic renal failure, showing that increased water intake or chronic infusion of a VP V2-receptor antagonist reduced proteinuria and prevented glomerulosclerosis[6;9] and tubulo-interstitial fibrosis.[10] Similarly, chronic infusion of a V2-receptor antagonist reduced albuminuria and kidney weight in diabetic rats.[11]
Interest in pleiotropic effects of VP has risen because VP antagonists are expected to become available for clinical use within the next years. Human studies showing deleterious effects of VP on renal function are, however, lacking. Presumably, this is caused by the fact that direct measurement of VP in human is problematic. More than 90% of VP in the circulation is bound to platelets, VP is unstable in isolated plasma,[12] and most VP assays have relatively limited sensitivity. Recently, an assay has been developed to measure copeptin, the C-terminal portion of the precursor of VP. Copeptin has been shown to be a reliable marker of VP secretion and a useful substitute for circulating VP concentration in clinical routine.[13-15] These characteristics make copeptin a promising marker, which allows for the first time (indirect) measurement of VP in a large population.
Renal transplant recipients often exhibit renal function deterioration and have a higher VP concentration than healthy controls.[16] Because of the aforementioned hypothetical deleterious effects of VP, we hypothesized that a high concentration of copeptin is associated with renal function decline during follow- up in renal transplant recipients and investigated this hypothesis in a prospective observational study.

Materials and Methods
Study Design and Patients
In this prospective observational study, all renal transplant recipients who visited our outpatient clinic between August 2001 and July 2003 and had a functioning graft for at least 1 year were eligible to participate at their next visit to the outpatient clinic (baseline). Baseline visits were postponed until symptoms had resolved in patients with fever or other signs of infection (e.g. complaints of upper respiratory tract infection or urinary tract infection). Patients with overt heart failure and patients diagnosed with cancer other than cured skin cancer were not considered eligible for the study. A total of 606 of 847 (72%) eligible renal transplant recipients signed written informed consent. The group that did not sign informed consent was

comparable with the group that signed informed consent concerning age, gender, body mass index (BMI), baseline serum creatinine, creatinine clearance, and proteinuria. All participating subjects visited the outpatient clinic at least once a year, and serum creatinine was assessed at every visit. To assess change in renal function over time, baseline renal function was related to information on renal function obtained at the last known visit to the outpatient clinic. Follow-up date for patients who died with a functioning graft (n=32) was defined as renal function at the last visit to the outpatient clinic before death, and follow-up for patients with graft failure (n=17) was defined as renal function at the last visit to the outpatient clinic before starting dialysis. Details of this study have been published previously.[17;18] Excluded from our study were recipients without copeptin value at baseline (n=28, 4.6%, because of missing samples) and recipients with a follow-up of less than 1 year (n=30), leaving a total of 548 recipients for analysis. The institutional review board approved the study protocol (METc 01/039), which was in adherence to the declaration of Helsinki. Funding sources had neither a role in the collection and analysis of data, nor in the submission and publication of the manuscript.

Immunosuppressive Medication

Standard immunosuppression consisted of the following: from 1968 until 1989, prednisolone (10 mg/day) and azathioprine (100 mg/day). From 1989 until 1993, cyclosporine standard formulation (Sandimmune; Novartis, Huningue, France; 10 mg/kg; trough concentrations of 175–200 µg/L in first 3 months, 150 µg/L between 3 and 12 months post transplant and 100 µg/L thereafter) combined with prednisolone (starting with 20 mg/day, rapidly tapered to 10 mg/day). From 1993 until 1996, cyclosporine micro emulsion (Neoral; Novartis Pharma b.v. Arnhem, The Netherlands; 10 mg/kg; trough concentrations idem) and prednisolone. From May 1996 until present, mycophenolate mofetil (Cellcept; Roche b.v., Woerden, The Netherlands; 2 g/day) was added to the latter regimen. Current medication was extracted from the medical record of individual patients.

Baseline Measurements and Definitions

The BMI was calculated as weight in kilograms (kg) divided by height in square meters (measured to the nearest 0.5 kg and 0.5 cm, respectively). Blood pressure was measured as the average of three automated measurements (Omron M4; Omron Europe B.V. The Netherlands) with 1-min interval after a 6-min rest in supine position. Mean arterial pressure (MAP) was calculated using the standard formula: 2/3 diastolic blood pressure +1/3 systolic blood pressure. Plasma glucose was determined by the glucose-oxidase method (YSI 2300 Stat plus, Yellow Springs, OH). Diabetes mellitus was diagnosed if fasting plasma glucose concentration was more than or equal to 7.0 mmol/L and antidiabetic medication was used. Serum sodium, potassium, urea, uric acid, and urinary urea were determined with dry chemistry, using a standard auto-analyzer (Kodak Ektachem; Eastman Kodak, Rochester, New York). Serum creatinine and urinary creatinine concentration were determined using the Jaffe′ method (MEGA AU 510; Merck Diagnostica, Darmstadt, Germany). Because urea is an ineffective osmole that readily crosses cell membranes, it will not induce water movement out of the osmoreceptor cells,[19] and consequently it will not affect VP secretion. We, therefore, calculated effective plasma osmolarity and not total osmolarity. Effective plasma osmolarity was calculated as plasma osmolarity=2 x concentration sodium + concentration glucose.[19] Fractional urea excretion was calculated as FE urea= ([urinary urea concentration/plasma urea concentration]/[urinary creatinine concentration/ plasma creatinine concentration]) x100. Total protein concentration was analyzed using the Biuret reaction (MEGAAU 510; Merck Diagnostica, Darmstadt, Germany).

N-terminal pro brain natriuretic peptide (NTproBNP) measurements were performed in plasma on an Elecsys 2010 analyzer, a commercially available electrochemiluminescent sandwich immunoassay (Elecsys proBNP; Roche Diagnostics, Mannheim, Germany). Creatinine values were used to calculate estimated Glomerular Filtration Rate (eGFR) at baseline and at follow-up, using the Modification of Diet in Renal Disease (MDRD) formula.[20] The assay of human leukocyte antigen classes I and II antibodies was performed by ELISA (LATM20X5, One Lambda, Canoga Park, CA); positive and negative samples were identified according to the instructions of the manufacturer.

The eGFR at follow-up was subtracted from that of baseline, and this value was divided by time from baseline to follow-up, to obtain a delta eGFR in mL/min/1.73 m2 per year. This delta eGFR (change in renal function over time) is our outcome variable for the longitudinal analyses. Our main study variable, copeptin was measured in samples stored at -80°C, that were transported in frozen condition to the laboratory of Brahms Berlin, using a new sandwich immunoassay (B.R.A.H.M.S. AG, Hennigsdorf/Berlin, Germany), as described previously.[13] Briefly, this sandwich immunoluminometric assay uses two polyclonal antibodies to the C-terminal region (acid sequence 132–164) of prepro-AVP. One antibody is bound to polystyrene tubes and the other is labeled with acridinium ester. The assay requires 50µL of plasma and no extraction steps or other preanalytical procedures.

Statistical Analysis

Analyses were performed with SPSS version 16.0 (SPSS Inc., Chicago, IL). Normality was tested with the Kolmogorov-Smirnov test. Parametric variables are expressed as mean±SD, whereas nonparametric variables are given as median (interquartile range). A two-sided P value less than 0.05 was considered to indicate statistical significance.

To investigate potential associations with copeptin and other variables (plasma osmolarity, 24 hr urinary volume, urinary sodium concentration, and fractional urea excretion) at baseline, univariate linear regression analyses were performed. To visualize these associations, we divided copeptin in tertiles of increasing copeptin concentration. Because men have a significant higher copeptin concentration than women,[14] these tertiles were stratified for gender. Statistical testing of differences between the tertiles was performed with ANOVA (when the variable was normally distributed) and Kruskal-Wallis (in case of skewed distribution).

For the analyses of copeptin as predictor of change in renal function during follow-up, we performed continuous analyses by performing univariate regression analyses with baseline log copeptin as independent variable and change in renal function as the dependent variable (Table 2, model 1). To visualize this association, we compared rate of renal function decline for gender-stratified tertiles of copeptin. Subsequently, we used univariate regression analysis, to investigate the association between copeptin and change in renal function, using copeptin as a continuous

variable. Using a multivariate regression model, this association was adjusted for several factors that could potentially be confounders in this association. We built multivariate models stepwise. First, our association was adjusted for gender and age (model 2). Second, baseline eGFR was entered into the model (model 3). In addition to that, factors known from literature that could possibly influence VP (and thus copeptin) secretion (osmolarity, BMI, Diabetes, and use of diuretics and of angiotensin converting enzyme inhibitors/angiotensin II receptor blockers) were added into the model (model 4).[19] Because heart failure and inflammation could underlie both elevated copeptin concentration and renal function decline, the model was also adjusted for C-reactive Protein (CRP) and NTproBNP (model 5). Finally, in addition to the previous steps, we also adjusted for other known determinants of renal function decline (time on dialysis before kidney transplantation
(ntx, time from ntx until baseline, living related ntx, donor age, immunosuppressive medication, antidonor antibody status, current smoking, MAP, and proteinuria) (Model 6). Because borderline positive antidonor antibodies seem to have protective value, we added the antibody status as categorical variable in the model. For analyses, immunosuppressive medication was subdivided into three groups: prednisolone, calcineurin inhibitors (tacrolimus/cyclosporin), and proliferation inhibitors (azathioprine/mycophenolate mofetil). Because all subjects use prednisolone, daily dose was entered as a continuous variable. We tested for significant interactions between patient characteristics and baseline log copeptin in predicting change in renal function during follow-up.

Various sensitivity analyses were performed. Because use of diuretics influences copeptin concentration and possibly also are associated with renal function decline, the longitudinal analyses were also performed separately for subjects with and without diuretics use. Despite the fact that no significant interaction was found, we also repeated the analyses stratified for BMI (in tertiles), gender, renal function (increments of 10 mL/min), diabetes mellitus status (yes/no), and with and without outliers of copeptin concentration (copeptin >21.8 pmol/L for women, n=30 and copeptin>39.5 pmol/L for men, n=21). To investigate possible survivor bias, we added year of transplantation as an interaction term in the multivariate analysis and performed a sub analysis in patients who were between 1 and 3 years after donation (n=143). Our primary outcome variable is the difference between initial eGFR and last recorded eGFR. We also repeated our analyses using slopes through the four eGFR values over time that are available in our database (baseline, and at 2.4, 2.8, and 3.1 years after baseline).

Results

Patient characteristics are presented in Table 1. A total of 548 patients (54% men, aged 52±12 years at baseline) were analyzed. Median time between transplantation and baseline measurements was 6.0 (2.7–11.6) years. Median copeptin concentration for the whole group at baseline was 9.1 (5.0– 18.5) pmol/L. Median copeptin concentration was 12.2 pmol/L (interquartile range 6.3–23.3) for men and 6.6 pmol/L (4.1–12.7) for women (P=0.001). The distribution of copeptin values ranged from 1.4 to 102.0 pmol/L.

To investigate whether copeptin concentration, as measure of VP, is consistent with normal physiological regulation, we tested the association between plasma osmolarity, one of the causes of VP secretion and copeptin. We found that plasma osmolarity was positively associated with log copeptin (R=0.13, P=0.003). Figure 1 shows that from tertile 1 to tertile 3 effective plasma osmolarity is increasing, indicating that indeed a higher plasma osmolarity is associated with a higher copeptin concentration.

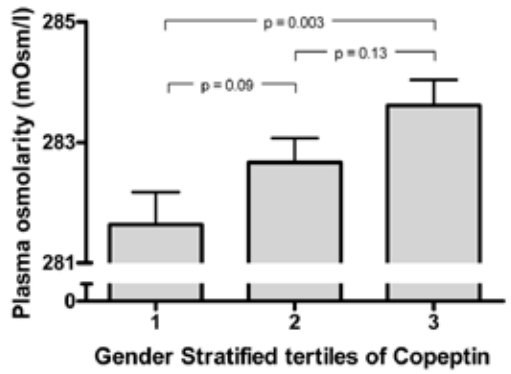


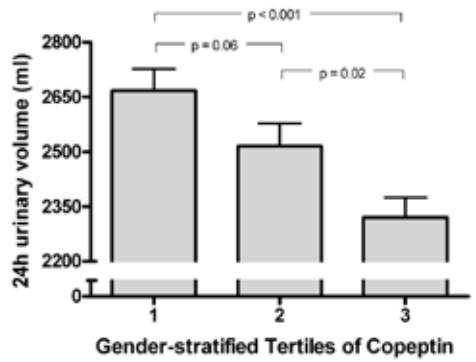
Figure 1: Physiologic regulation of vasopressin: Mean (± SE) effective plasma osmolarity per gender-stratified tertile of copeptin

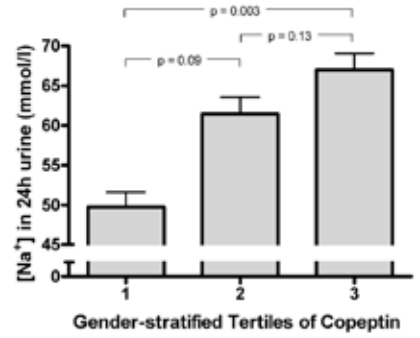
Table 1. Patient characteristics at baseline

Variable	*Value*
Age (years)	52 ±12
Male gender n (%)	296 (54)
Prior history of cardiovascular disease n (%)	68 (12)
Current Smoking n (%)	117 (21)
BMI (kg/m2)	26.2 ± 4.3
Mean arterial pressure recipient (mm Hg)	111 ± 13
Blood pressure lowering drugs	
- Diuretics n (%)	238 (43)
- ACEi / ARB n (%)	186 (34)
Diabetes at baseline n (%)	111 (20)
Time from ntx until baseline (years)	6.0 (2.7-11.6)
Time on dialysis prior to ntx (months)	27 (14-48)
Living related donation n (%)	49 (9)
Donor age (years)	37 ± 16
Immunosuppressive regimen	
- Calcineurin inhibitor (tacrolimus/ciclosporin) n (%)	433 (79)
- Proliferation Inhibitor (azathioprine / MMF) n (%)	406 (74)
- Prednisolone n (%)	548 (100)
Anti-donor antibody status	
- Negative, class I n (%) / class II n (%)	480 (88) / 481 (88)
- Borderline, class I n (%) / class II n (%)	20 (4) / 11 (2)
- Positive, class I n (%) / class II n (%)	48 (9) / 56 (10)
Serum potassium (mmol/l)	4.5 ± 0.5
Serum sodium (mmol/l)	139 ± 3
Effective plasma osmolarity (mOsm/l)	283 ± 6
Serum uric acid (mmol/l)	0.45 ± 0.12
NT pro-BNP (pg/ml)	285 (129-622)
Urinary volume (l/24h)	2.5 ± 0.8
Sodium excretion (mmol/24h)	139 ± 62
Urea excretion (mmol/24h)	381± 113
Fractional urea excretion (%)	45 ± 11
Proteinuria (g/24h)	0.2 (0.0-0.5)
eGFR (ml/min/1,73m2)	47 ± 14
Copeptin (pmol/l)	9.1 (5.0-18.5)

N = 548. Parametric variables are expressed as mean ± SD, whereas non-parametric variables are given as median (interquartile range). Abbreviations are: BMI, body mass index; ACEi, angiotensin converting enzyme inhibitors; ARB, angiotensin II receptor blockers; ntx, kidney transplantation; NT pro BNP, N-Terminal pro Brain Natriuretic Peptide; eGFR, estimated Glomerular Filtration Rate.

To investigate whether the effects of VP are consistent with normal physiology, we tested the associations between copeptin and 24-hr urinary volume, urinary sodium concentration and fractional urea excretion. A high concentration of copeptin was found to be associated with a low 24-hr urinary volume (R=-0.22, P =0.001), low fractional urea excretion(R= -0.28, P=0.001), and a high urine (sodium) concentration (R=0.33, P=0.001). Figure 2 visualizes these associations.





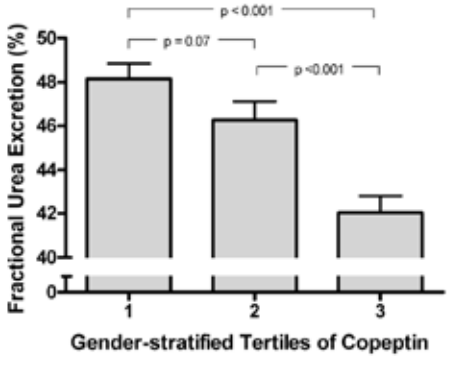


Figure 2. Physiologic effects of vasopressin: Mean (± SE) 24h urinary volume, urinary sodium excretion, and fractional urea excretion per gender-stratified tertile of copeptin

Median (interquartile range) follow-up after baseline was 3.2 (2.7–3.7) years. Mean change in renal function over this period was -0.52 mL/min/1.73 m2 per year. During follow-up, a significant association was found between baseline log copeptin and change in renal function (R= -0.14, P=0.001). This inverse association indicates that a high log copeptin concentration at baseline was associated with more renal function decline during follow-up.

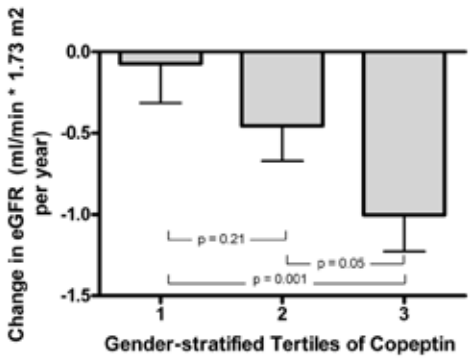


Figure 3. Mean (± SE) change in renal function according to gender-stratified tertiles of copeptin

In Figure 3, mean change in renal function during follow-up is depicted per gender-stratified tertile of copeptin. Duration of follow-up was the same for the three tertiles; 3.2 (2.6 –3.6) years, 3.3 (2.9 –3.7) years, and 3.2 (2.7– 6.6) years, respectively (P=0.99). Mean change in renal function over time in tertiles 1, 2, and 3, respectively, was -0.03(±3.20), -0.44 (±3.01), and -1.06 (±3.00) mL/min/1.73 m2 per year.

The association between baseline log copeptin and change in renal function during follow-up was also investigated using copeptin as a continuous variable in multivariate regression analyses. We adjusted this association in a multivariate model for several potential confounders. Results are listed in Table 2. The association was significant (with β=-1.2, P=0.001, Table 2, model 1). This indicates that an increase of logcopeptin with 1 unit, parallels with a renal function decline of 1.2 mL/min/yr). Adjusted for age and gender, the association remained significant. Further adjustment for baseline eGFR (model 3), for factors that might influence VP and thus copeptin concentration (model 4), and for factors influencing renal function decline (model 6) did not materially influence the association between log copeptin and change in renal function during follow-up. The r value for the association of log copeptin with renal function is -0.14. This is, in this population, at least comparable with other acknowledged factors of renal function decline, as for instance for the association between baseline mean arterial pressure and delta renal function, for baseline glucose, for age, and for baseline renal function.

Table 2. Associations between baseline log copeptin concentration and change in renal function during follow-up.

model	*corrected for*	*β*	*P*
1	- (crude)	-1.2	0.001
2	As 1 + age and gender	-1.2	0.001
3	As 2 + baseline eGFR	-1.8	<0.001
4	As 3 + BMI, plasma osmolarity, diabetes, use of diuretics and ACEi / ARB	-1.9	<0.001
5	As 4 + NT pro BNP and CRP	-1.7	<0.001
6	As 5 + time on dialysis before ntx, time from ntx until baseline, living related ntx, donor age, immunosuppressive medication, anti-donor antibody status, current smoking, MAP and proteinuria.	-1.5	0.003

A negative beta indicates that a high baseline copeptin concentration is associated with more renal function decline during follow-up.

Because it has been hypothesized that VP is involved in causing hypertension,[21] a regression analysis with mean arterial pressure as the dependent variable and log copeptin as the independent variable was performed. This association was not significant when analyzed univariate (P=0.22), or when corrected for the use of antihypertensive medication (P=0.33), or when corrected for use of such medication and age and gender (P=0.35). Of note, the results we obtained in the various sensitivity analyses were essentially similar to those from our primary analyses. No significant interaction was found between gender and baseline log copeptin in predicting change in renal function during follow-up, meaning that the association between baseline log copeptin and change in renal function is not different for men or women. Also no interaction was found between renal function at baseline and log copeptin in predicting renal function decline. When analyses were stratified for BMI (tertiles), gender, renal function (increments of 10 mL/min), or diabetes mellitus (yes vs. no), the main results were similar to those of our primary analysis. The same holds true when the analyses were repeated with and without outliers with respect to copeptin concentration, when a subgroup analysis was performed in patients only between 1 and 3 years after donation (indicating that survivor bias does not play an important role), or when slope of renal function was used as the primary outcome.

Discussion

In this study, we aimed to investigate the possible association between copeptin concentration at baseline and change in renal function during follow-up in a cohort of renal transplant recipients. Regulation and action of copeptin in this study seemed to be consistent with normal physiology because we found, at baseline, a positive association between plasma osmolarity and copeptin concentration, a negative association between copeptin and 24 hr urinary volume and fractional urea excretion, and a positive association between copeptin and urinary sodium concentration.

With respect to our primary study question, we found that an elevated concentration of copeptin was associated with accelerated renal function decline in these renal transplant recipients. This association seemed to be independent of baseline eGFR, proteinuria, and of other known risk factors for renal function decline in renal transplant recipients. As far as we know, this is the first human study to investigate the association between VP concentration (measured as copeptin) and changes in renal function during follow-up. It confirms the results obtained so far in animal experiments. Furthermore, this study has been performed in a large number of subjects and had sufficient duration of follow-up (more than 3 years).

This study found copeptin concentration in renal transplant recipients to be approximately twofold higher in men (median concentration 12.2 pmol/L) than in women (6.6 pmol/L), in agreement with previous reports,[14;22] and consistent with a gender difference in plasma VP and in urinary osmolarity.[23] This copeptin concentration is higher than the values of 5.2 and 3.7 pmol/L, for men and women, respectively, that have been reported in healthy controls.14 In renal transplant patients, VP concentration is previously been described to be higher than in healthy controls.16 A reason for this difference could be that renal transplant recipients have lower renal function than healthy controls (mean eGFR in this study is 47±14 mL/min/1.73 m2). Renal function has previously been described to be associated with copeptin concentration.[22] The reason for this phenomenon is not known. It could be hypothesized that copeptin concentration is a consequence of less renal clearance because VP (and also copeptin) is cleared for about 25% by the kidneys.[24] This hypothesis is, however, less likely because negative feedback mechanisms should reduce the rate of secretion of the hormone and bring it back to normal concentration (assumed that the effect on the target organ is not impaired).

Because of the cross-sectional association between copeptin and renal function, renal function could be a confounder for the association between copeptin and change in renal function. Therefore, we corrected for baseline eGFR in our multivariate regression analysis. After this adjustment, copeptin remained significantly associated with the rate of renal function decline during follow-up (Table 2, model 3, etc.). Furthermore, we repeated multivariate analysis stratified for eGFR, which did not materially influence our results.

Because we wanted to investigate whether there are certain subgroups that are more susceptible to copeptin's influences, we investigated potential interactions between log copeptin on one hand and on the other gender, age, or baseline renal function. These interactions were not significant, suggesting that the association between log copeptin and rate of renal function loss during follow-up is essentially similar in men and women, in various age groups and independent of renal function. The observation that a high concentration of copeptin is associated with accelerated renal function decline gives rise to the hypothesis that a high-VP concentration is related to rate of renal function decline in these patients.

Animal experiments indeed suggest that a high concentration of VP causes accelerated renal function decline. A chronic reduction in plasma VP concentration slowed the progression of renal failure in Sprague-Dawley rats with impaired renal function,6 whereas infusion of 1-Desamino 8-D-arginine Vasopressin (dDAVP) induced a rise in serum creatinine and in proteinuria in Brattleboro rats.[7]

Human studies on the association between VP concentration and rate of renal function decline are scarce. To our knowledge only one study indirectly addressed this issue. A retrospective analysis of the MDRD study showed that, in patients with chronic kidney disease, higher urinary volume and lower estimated urinary osmolarity were associated with faster eGFR decline.[25] The authors offered two possible explanations for this relationship. The first is that excessive fluid intake may cause faster renal disease progression. This would be in contrast to our findings, because high fluid intake is probably associated with low concentration of VP. The other proposed possibility is that, instead of the cause, a high urinary volume with a low urinary osmolarity is the result of faster renal disease progression. This explanation might be more likely because one of the clinical manifestations of chronic renal failure is a defect in urinary concentrating ability.[26] Unfortunately, VP (or copeptin) concentration is not determined in this study, and definite conclusions could, therefore, not be drawn.

The unfavorable renal effects of VP have been argued to be partly due to an effect on blood pressure. VP may contribute to hypertension by a direct effect on vascular smooth muscle through activation of the V1a receptor[27] or by V2 receptor-dependent tubular effects. The latter includes an enhancement of sodium reabsorption in the collecting duct,[28] and an increase in intrarenal urea recycling that participates in the urine concentrating mechanism. The latter may indirectly modify NaCl concentration at the macula densa, thereby influencing tubuloglomerular feedback, leading to renin release, hypertension, glomerular hyperfiltration, proteinuria, and, if sustained, renal hypertrophy and tubulointerstitial disease.[8;10]

In our study, however, no association was found between copeptin concentration and blood pressure, and the association between baseline copeptin and rate of renal function loss during follow-up was independent of blood pressure, indicating that a rise in blood pressure by VP is not likely in our patients and that other mechanisms of action may be important. Also, unidentified factors could contribute simultaneously to a rise in VP secretion and to an accelerated renal function decline. Examples of such factors could be inflammation and heart failure. The pathologic condition of chronic transplant dysfunction probably includes inflammation.[29] Several studies have shown that proinflammatory cytokines can activate VP secretion.[30] Therefore, inflammation could induce both VP secretion and accelerated decline in renal function. Another factor could be cardiac failure, which might result in both high VP concentration[31] and accelerated renal function decline. However, in the previously mentioned animal experiments where VP administration led to renal function deterioration, and intervention with VP antagonists led to renoprotection, strongly suggest a causal role for VP and make the possibility of an underlying factor leading to both a rise in VP and accelerated renal function decline, less likely. Furthermore, the association between copeptin concentration and renal function decline also remained significant after adjustment for CRP and NT-proBNP (Table 2, model 5), markers of inflammation and severity of heart failure. Definite proof for a causal role of VP in inducing renal function decline should come from randomized intervention studies. At the moment, several selective (V1a or V2) and combined V1/V2 receptor antagonists are being developed for clinical use.[32] Use of these drugs may provide a valuable tool to determine whether VP is related to renal function decline, and whether the V1a and/or the V2 receptor are involved.

The association between copeptin and renal function decline has an r value of -0.14. This relatively low r value indicates that other factors also contribute to rate of renal function decline. We included several of the known risk factors for renal function decline in transplant recipients in our multivariate model (Table 2), for example, age, gender, baseline eGFR, diabetes, BMI, NT-proBNP (a surrogate for cardiac failure), CRP (a surrogate for subclinical infection), time on dialysis before ntx, time from ntx until baseline, living related ntx, donor age, immunosuppressive medication, antidonor antibody status, current smoking, MAP, and proteinuria, and the association between copeptin and renal function decline was still significant after adjustment for all these factors. Furthermore, it appeared that the r value of copeptin was one of the strongest and that the association between well acknowledged renal risk factors (e.g. blood pressure and fasting glucose) and the rate of renal function decline is of approximately similar or lower strength (r value is of similar strength).

We acknowledge that the present study has limitations. Because of the observational nature of our study, it is impossible to draw a definite conclusion about causality. We cannot exclude that other, yet unidentified factors, led to an elevated copeptin concentration and accelerated renal function decline. Furthermore, renal function is estimated using the MDRD formula, which is less precise than for instance iothalamate clearance, but more frequently used.

Our creatinine measurement method (Jaffe′) is not calibrated against IDMS traceable standards. However, because we calculated changes in eGFR, instead of only one value (and for all eGFR values of a given subject the same correction factor would have to be used), we expect that this would minimally influence our results. We measured copeptin at the beginning of the study once, instead of as average of three measurements this will probably have led to an underestimation of the effect size, given the widening of confidence intervals that would have occurred. Finally, the study population consisted of renal transplant recipients who were almost all of white ethnicity. Whether our results can be extrapolated to other populations, such as subjects with impaired renal function without transplantation or subjects of other ethnicity, is therefore unknown.

In conclusion, copeptin (a surrogate marker of VP) is independently associated with rate of renal function decline during follow-up in renal transplant recipients. This finding suggests that VP may play a role in renal function decline. This alludes to the intriguing possibility that interventions leading to lower VP activity, as drinking more water or using the newly developed VP receptor antagonists, may be beneficial for the prevention of renal function decline in these patients.

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Part II

Chapter 6

Copeptin, a surrogate marker of vasopressin, is associated with disease severity in Autosomal Dominant Polycystic Kidney Disease.

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Abstract
Experimental studies suggest a detrimental role for vasopressin in the pathogenesis of Autosomal Dominant Polycystic Kidney Disease (ADPKD). It is, however, unknown whether endogenous vasopressin concentration is associated with disease severity in patients with ADPKD.
We measured plasma copeptin concentration (a marker of endogenous vasopressin levels) in 102 ADPKD patients (diagnosis based on Ravine criteria) by an immunoassay. Plasma- and urinary osmolarity were also measured. To assess disease severity, we measured glomerular filtration rate and effective renal blood flow by continuous infusion of 125I-Iothalamate and 131I-Hippuran, total renal volume by MRI and 24h urinary albumin excretion by nephelometry.
In these ADPKD patients (age 40±11y, 56% male, GFR 77±31 ml/min per 1.73 m2, total renal volume 1.5 (0.9-2.2)L), copeptin was associated with the various markers of disease severity in ADPKD (positively with total renal volume (R=0.47) and albuminuria (R=0.39) and negatively with glomerular filtration rate (R=-0.58) and effective renal blood flow (R=-0.52), all $p<0.001$). These associations were independent of age, gender and use of diuretics. Copeptin was furthermore associated with plasma osmolarity ($p<0.001$), but not with 24h urinary volume, 24h urinary osmolarity or fractional urea excretion ($p=0.7$, $p=0.9$, $p=0.3$, respectively).
On cross-sectional analysis, copeptin is associated with disease severity in ADPKD patients, supporting the results of experimental studies that suggest that vasopressin antagonist have a renoprotective effect in ADPKD, and offering a good prospect for clinical studies with these agents.

Introduction
Autosomal Dominant Polycystic Kidney Disease (ADPKD) is the most common renal hereditary disease (caused in most cases by a mutation in the PKD1 or PKD2 gene[1,2]) with an incidence of 1 in 400 to 1,000 live births.[3,4] The disease is characterized by progressive cyst formation in both kidneys, often leading to end stage renal disease. Current treatment is not able to inhibit cyst formation or to prevent renal failure.
Vasopressin, also known as antidiuretic hormone, is essential for regulation of water homeostasis and osmoregulation in the body. Its secretion is stimulated in response to an increase in plasma osmolarity or a decrease in blood volume. Vasopressin binds to the V2 receptors in the collecting duct, which induces the insertion of the molecular water channel aquaporin-2 in the luminal membrane of principal cells. This mediates water resorption, thereby reducing water excretion.[5]
Despite its role for normal physiology, vasopressin has also been mentioned to be involved in pathophysiological processes, among others in ADPKD. Vasopressin promotes cAMP production by acting on vasopressin-2 receptors in the distal nephron and collecting ducts. Experimental studies have implicated a central role for 3-5-cyclic adenosine monophosphate (cAMP) in promoting cyst growth.[6] cAMP stimulates cyst formation by promoting chloride-driven fluid secretion and by stimulating activation and proliferation of cyst-derived cells.[7] In line with this potential detrimental role of vasopressin are the findings in animal models of polycystic kidney disease, where blocking the effect of vasopressin (and consequently decreasing cAMP levels) by either a pharmacological agent[8-10] or by drinking more water[11] led to reduction of cyst formation and renal function preservation.
So far, no studies have looked at the association between endogenous vasopressin levels and disease severity in subjects with ADPKD. The paucity of data on this issue may be caused by the fact that measurement of vasopressin is problematic. More than 90% of VP in the circulation is bound to platelets, VP is unstable in isolated plasma,[12] and most VP assays have relatively limited sensitivity. Recently, an assay has been developed to measure copeptin, the C-terminal portion of the precursor of VP. Copeptin has been shown to be a reliable marker of VP secretion and a useful substitute for circulating VP concentration in clinical routine.[13-15]
Because of the potential detrimental role of vasopressin in the pathogenesis of ADPKD, we aimed to investigate whether endogenous copeptin levels (a surrogate marker of vasopressin) are associated with disease severity in subjects with ADPKD. As markers of disease severity we studied glomerular filtration rate, effective renal blood flow, total renal volume and albuminuria. To investigate whether copeptin concentration is consistent with normal physiological regulation and has its normal physiologic effects, we also studied associations with plasma osmolarity, 24h urinary volume, 24h urinary osmolarity and fractional urea excretion.

Materials and Methods
ADPKD patients
One hundred and twenty consecutive patients with ADPKD visiting our out-patient clinic meeting our in- and exclusion criteria were asked to participate. Diagnosis of ADPKD was made upon Ravine criteria.[16] Subjects were considered ineligible to participate if they received renal replacement therapy, had undergone renal surgery, were unable to undergo magnetic resonance

imaging (as having distorting foreign bodies or aneurysmal clips), had other systemic diseases potentially affecting renal function (as diabetes mellitus and malignancies), or had other medical conditions that included pregnancy, lactation, or who were less than 6 months postpartum.
After screening, subjects underwent an extensive medical history. Subjects were scheduled for a 1-day outpatient clinic evaluation. Thirteen patients refused to participate and two patients were not eligible. Three patients had a copeptin concentration more than 10 times the interquartile range above the third quartile, although their plasma osmolarity was within normal limits. These subjects were considered outliers[17] and their data were not taken into consideration, leaving 102 patients for analyses.
This study was performed in adherence to the declaration of Helsinki. All subjects gave written informed consent.

Measurements and definitions

Blood pressure was assessed with an automatic device (Dinamap) for 15 minutes during the renal function measurement. Systolic- and diastolic blood pressure values were used to calculate mean arterial pressure (MAP) using the standard formula: 2/3 diastolic blood pressure + 1/3 systolic blood pressure. Weight and height were determined. Body mass index (BMI) was calculated as weight in kilograms (kg) divided by height in square meters. Patients collected a 24h urine sample prior to the outpatient visit. Urinary albumin concentration was determined by immuno nephelometry (BNII; Dade Behring Diagnostics, Marburg, Germany). Prior to renal function measurement, blood samples were drawn for determination of haemoglobin, sodium, creatinine, urea, plasma osmolarity and copeptin. Concentrations of haemoglobin, sodium and urea were measured using standard methods. Creatinine was measured with the Roche enzymatic creatinine assay. Plasma- and urine osmolarity was measured using freezing point depression. Fractional urea excretion was calculated as FE Urea = ((urinary urea concentration / plasma urea concentration) / (urinary creatinine concentration / plasma creatinine concentration)) * 100.

Copeptin was measured using a new sandwich immunoassay (B.R.A.H.M.S. AG, Hennigsdorf / Berlin, Germany), which was based on the assay described previously13. The assay was modified as follows: the capture antibody was replaced by a murine monoclonal antibody directed to amino acids 137-144 of proAVP. This modification improved the sensitivity of the assay. The lower detection limit was 0.4 pmol/L and the functional assay sensitivity (20% interassay coefficient of variation) was less than 1 pmol.[18]
Renal function measurements were performed using the constant infusion method with 125I-Iothalamate to measure glomerular filtration rate (GFR) and with 131I-Hippuran to measure effective renal plasma flow (ERPF).[19-22] Effective renal blood flow (ERBF) was calculated as ERPF / (1-Hematocrit). Hematocrit was measured halfway during the renal function measurement.)
Patients underwent a standardized abdominal magnetic resonance imaging protocol without the use of i.v. contrast. Scanning was performed on a 1.5 Tesla MRI Magnetom Avento (Siemens, Erlangen, Germany) with the use of body matrix and spine matrix coils. Total renal volume (TRV) was measured on T2 weighted coronal images[23] (slice-thickness 4.0 mm) using Analyze

Direct 8.0 (AnalyzeDirect, Inc., Overland Park, KS) software.

Statistical analyses

Analyses were performed with SPSS version 16.0 (SPSS Inc., Chicago, IL). Parametric variables are expressed as mean ± standard deviation (SD), whereas non-parametric variables are given as median (interquartile range). A two sided $p < 0.05$ was considered to indicate statistical significance.
Regression analysis was performed to investigate whether plasma copeptin concentration was correlated with plasma osmolarity, physiologic variables and variables representing disease severity. In case of non-normal distribution, variables were log transformed, and R and p values are given. GFR and ERBF were normalized for body surface area. To visualize the associations, scatter plots (with males and females depicted separately) were made showing the associations between copeptin and plasma osmolarity and markers of disease severity (TRV, GFR, ERBF and albuminuria).
To further investigate whether plasma copeptin concentration was associated with markers of disease severity, multivariable regression analysis was performed. Logarithmic transformation of copeptin (and of urinary albumin excretion and total renal volume) was applied to fulfil the requirement of equal distribution of the residuals. Associations were investigated crude and after adjustment for age, gender, use of diuretics and GFR.
Interactions between log copeptin concentration and age and gender were tested for GFR, ERBF, log total renal volume and log UAE as the dependent variables.

Results

Characteristics of participating patients are depicted in Table 1. A total of 102 patients (56% male, aged 40± 11 years) were analyzed. Median copeptin concentration for the whole group was 7.0 (3.1-15.7) pmol/L. Median copeptin concentration was higher in men than in women (10.1 (4.7-19.8) vs. 3.2 (2.4-8.3) pmol/L, p<0.001).

Variable	
Men, n (%)	57 (56)
Age (y)	40 ± 11
Body mass index (kg/m^2)	26 ± 5
Mean arterial pressure (mm Hg)	96 ± 10
Use of antihypertensive medication, n (%)	79 (78)
Use of diuretics, n (%)	23 (23)
Haemoglobin (mmol/l)	8.3 ± 0.8
Serum sodium (mmol/L)	140 ± 2
Plasma osmolarity (mOsm/L)	290 ± 8
Copeptin (pmol/L)	7.0 (3.1-15.7)
Serum creatinine (µmol/L)	115 ± 70
Serum urea (mmol/L)	8 ± 4
24h urinary volume (L)	2.3 ± 0.8
24h urinary osmolarity (mOsm/L)	425 ± 148
24h urinary albumin excretion (mg/24h)	41 (15-122)
Fractional urea excretion (%)	42 ± 10
Glomerular filtration rate (ml/min/1.73 m^2)	77 ± 31
Effective renal blood flow (ml/min/1.73 m^2)	421 ± 170
Total renal volume (L)	1.50 (0.94-2.18)

Table 1: *Patient characteristics (n=102)*

Plasma copeptin concentration, as a surrogate of vasopressin, was significantly correlated with plasma osmolarity (figure 1, R=0.53, p<0.001). This correlation is not different for males when compared to females. No association was found between copeptin and urinary osmolarity (R= 0.01, p=0.9), copeptin and urinary volume (R= -0.04, p=0.7) or copeptin and fractional urea excretion (R= -0.10, p=0.3).

Figure 1: Association between plasma osmolarity and copeptin (overall R=0.53, p<0.001. Males ▲ and ▬, R=0.40, p=0.003, and females o and - -, R=0.46, p=0.002).

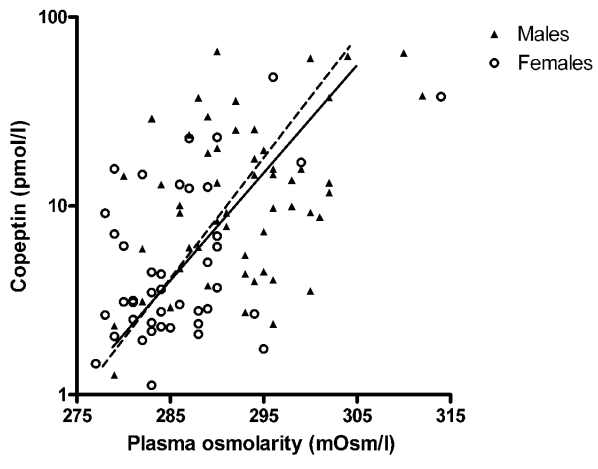

Table 2: Associations between the different markers of disease severity

	GFR		*ERBF*		*TRV*	
	R	p-value	R	p-value	R	p-value
ERBF	0.94	<0.001	-	-		
TRV	-0.42	<0.001	-0.30	0.002	-	-
UAE	-0.20	0.04	-0.15	0.1	0.47	<0.001

Abbreviations are: GFR, glomerular filtration rate; BSA, body surface area; ERBF, effective renal blood flow; TRV, total renal volume; UAE, 24h urinary albumin excretion. Depicted values are Pearson correlation coefficients and p-values. Variables are log transformed in case of non-normal distribution (total renal volume, urinary albumin excretion).

In general, the different markers of disease severity correlated well with each other. Correlation coefficients are depicted in Table 2. Looking into the association between copeptin and markers of disease severity, copeptin was positively associated with total renal volume (overall R=0.47, p<0.001, males R=0.47, p<0.001, females R=0.37, p=0.01) and 24h urinary albumin excretion (overall R=0.39, p<0.001, males R=0.30, p=0.03, females R=0.29, p=0.07 for females).

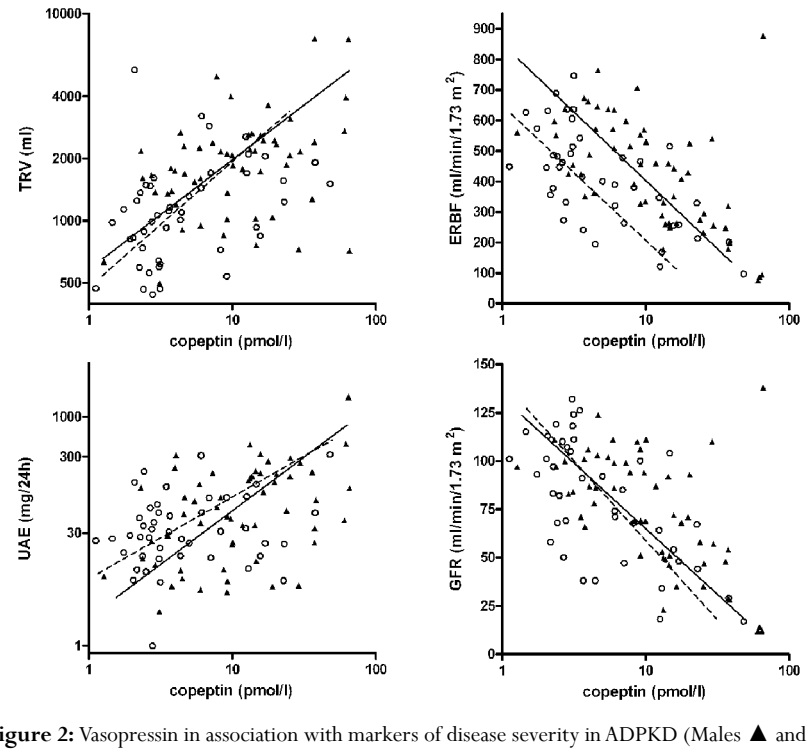

Figure 2: Vasopressin in association with markers of disease severity in ADPKD (Males ▲ and ▬. Females o and - -). On the left: associations between copeptin and total renal volume (TRV, upper panel, overall: R=0.47, p<0.001, males: R=0.37, p=0.006, females: R=0.37, p=0.01) and 24h urinary albumin excretion (UAE, lower panel, overall: R=0.39, p<0.001, males: R=0.30, p=0.03, females: R=0.29, p=0.07). On the right: associations between log copeptin and effective renal blood flow (ERBF, upper panel, overall R=-0.52, p<0.001, males: R=-0.57, p<0.001, females: R=-0.61, p<0.001) and glomerular filtration rate (GFR, lower panel, overall R=-0.58, p<0.001, males: R=-0.58, p<0.001, females: R=-0.61, p<0.001).

These associations remained significant after adjustment for age, gender and use of diuretics, and after adjustment of GFR (Table 3). In Figure 2, these associations are shown. Copeptin was furthermore inversely associated with effective renal blood flow (overall R= -0.52, p<0.001, males R= -0.57, p<0.001, females R= -0.61, p<0.001) and with glomerular filtration rate (overall R= -0.58, p<0.001, males R= -0.58, p<0.001, females R= -0.61, p<0.001). These associations remained significant after adjustment for age, gender and use of diuretics (Table 4). Correlation between effective renal blood flow and glomerular filtration rate was very high (R=0.94, p<0.001), therefore adjustment of the association between copeptin and ERBF for GFR was not possible. Figure 2 shows the associations between copeptin and GFR and ERBF. That females have a lower ERBF than males is at least partly caused by the known gender difference in hematocrit (also found in these patients, Ht was 0.41±0.03 for males vs. 0.38±0.03 for females, p<0.001) for estimated renal plasma flow is not different between males and females (253±106 vs. 256±97 ml/min per 1.73 m2 respectively, p=0.9).

Table 3: Associations between copeptin and total renal volume (TRV) and 24h urinary albumin excretion (UAE) as markers of disease severity in ADPKD.

Model	*Adjusted for*	*TRV*		*UAE*	
		Standardized β	p-value	Standardized β	p-value
1	crude	0.47	<0.001	0.39	<0.001
2	As 1 + age and gender	0.34	0.001	0.42	<0.001
3	As 2+ use of diuretics	0.33	0.001	0.44	<0.001
4	As 2 + GFR	0.23	0.05	0.26	0.04

Independent variable is log copeptin. Dependent variables are log total renal volume and log albuminuria. A positive standardized beta indicates that a high copeptin concentration is associated with a high TRV or UAE, respectively. Abbreviations are: TRV total renal volume; UAE 24h urinary albumin excretion.

Table 4: Associations between copeptin and glomerular filtration rate (GFR) and effective renal blood flow (ERBF) as markers of disease severity in ADPKD.

Model	*Adjusted for*	*GFR*		*ERBF*	
		Standardized β	p-value	Standardized β	p-value
1	crude	-0.58	<0.001	-0.52	<0.001
2	As 1 + age and gender	-0.55	<0.001	-0.55	<0.001
3	As 2 + use of diuretics	-0.53	<0.001	-0.54	<0.001

Independent variable is log copeptin. Dependent variables are GFR and ERBF (both in ml/min per 1.73 m2). A negative standardized beta indicates that a high copeptin concentration is associated with a low GFR or ERBF. Abbreviations are: GFR, glomerular filtration rate; BSA, body surface area; ERBF, effective renal blood flow.

Of note, we found no interactions between age and log copeptin concentration, or gender and log copeptin concentration on any of the aforementioned markers for disease severity (GFR, ERBF, total renal volume and albuminuria). The lack of interaction between gender and copeptin concentration on these markers is also shown in figures 1 and 2, where the regression lines for males and females all have the same slopes.

Discussion

In this study, we found that in ADPKD patients, plasma osmolarity was associated with copeptin concentration. Copeptin concentration however was not associated with 24h urinary volume, 24h urinary osmolarity or fractional urea excretion. Most importantly, we found that copeptin levels were associated with disease severity; higher copeptin levels were associated with lower renal function, lower effective renal blood flow, larger kidneys and more albuminuria. These associations were the same for males and females, and independent of age, and use of diuretics. The markers of disease severity that we studied are acknowledged in literature. Total renal volume is considered a good measure for disease severity because kidney enlargement results from the expansion of cysts in patients with ADPKD. Higher total renal volume is associated with a more rapid decrease in renal function.[24;25] In a large number of animal studies, treatments that inhibited renal enlargement, also ameliorated renal function. [25] Renal blood flow is also an accepted measure of disease severity. A reduction of renal blood flow parallels the increase in total renal volume, and importantly, precedes GFR decline and predicts structural and functional disease progression.[26] Several studies show that in ADPKD, albuminuria is associated with renal volume,[27;28] mean arterial blood pressure, filtration fraction,28 renal growth and slope of glomerular filtration rate.[29] In our study, the different markers for disease severity correlated reasonably well with each other.

Only two studies that we know of, both performed more than 10 years ago, measured vasopressin in ADPKD patients. One study described that vasopressin was increased ADPKD patients,[30] the other that vasopressin was increased in hypertensive ADPKD patients compared to non-hypertensive ADPKD patients and healthy controls.[31] These studies did not look into potential physiologic regulation and effects of vasopressin. In normal physiology, vasopressin is secreted in response to an increase in plasma osmolarity. We found that also in these ADPKD patients plasma osmolarity was associated with copeptin (as reliable and stable surrogate of vasopressin). Vasopressin mediates urea recirculation and water reabsorption and is therefore under normal circumstances positively associated with urinary osmolarity and inversely with urinary volume and fractional urea excretion. In these ADPKD patients however, we did not find significant associations between copeptin and 24h urinary volume, 24h urinary osmolarity or fractional urea excretion. This is in contrast to what is described in healthy subjects[32] and renal transplant recipients[33] and suggests that ADPKD patients do not respond effectively to vasopressin. In line with this assumption is the observation that ADPKD patients have an impaired urinary concentrating capacity, already present when GFR is still normal.[34]

We found that copeptin is associated with the various markers of disease severity in ADPKD. This association is consistent with the hypothesis that vasopressin is involved in disease progression in subjects with ADPKD. This hypothesis is supported by several experimental studies. Vasopressin, acting on V2 receptors, is described to increase cAMP in the distal nephron and collecting duct. This promotes chloride driven fluid secretion and, in polycystic kidney cells, stimulation of the B-RAF/MEK/extracellular signal-regulated pathway for mitogenesis and epithelial cell proliferation.[35;36] In line, we found an association between plasma copeptin

concentration and urinary cAMP excretion. Furthermore, a vasopressin V2 receptor antagonist inhibited cyst formation in models for ADPKD.[8;9] Moreover, genetic elimination of AVP in a rat model of ADPKD yielded animals born with normal kidneys that remained relative free of cysts unless an exogenous vasopressin V2 receptor agonist was administered.[37] We found copeptin to be higher in males than in females which is also consistent with the hypothesis that copeptin is involved in disease progression, for it is known that male gender is a risk factor for disease progression in ADPKD.[38-40]

Also in other renal conditions than ADPKD, vasopressin is thought to have a potential detrimental role. In non-ADPKD animal models, sustained vasopressin V2 receptor stimulation results in hyperfiltration,[41] which may have deleterious consequences, in particular in diseased kidneys, resulting in renal hypertrophy,[42] proteinuria,[43] and accelerated renal function decline.[44-46] In contrast, in rats with chronic renal failure, increased water intake (resulting in lower vasopressin concentration) or chronic infusion of a vasopressin V2-receptor antagonist reduced proteinuria and prevented glomerulosclerosis[44;47] and tubulo-interstitial fibrosis.[48] In humans, we have described previously that higher copeptin levels are associated with micro albuminuria in a population based cohort[32] and that higher copeptin levels at baseline predict renal function decline in renal transplant recipients.[33]

In our ADPKD patients, but also in non-ADPKD subjects,[32;49] copeptin concentration was found to be associated with renal function. The fact that copeptin is partly cleared by the kidney[50] is not likely to be an explanation for higher copeptin concentrations in subjects with lower GFR, because increased copeptin levels, would induce lowering of plasma osmolarity, which would lead to a reduced copeptin concentration. These mechanisms are difficult to dissect in a cross-sectional study. Alternatively, in subjects with lower GFR, the medullary urea gradient will be impaired due to loss of functioning nephrons and interstitial fibrosis. Subjects with impaired GFR have therefore lower urinary osmolarity and higher urinary volume than subjects with normal renal function[51;52] Consequently, fluid reabsorption will be more difficult and higher vasopressin levels will be necessary to maintain fluid balance. Furthermore, patients with ADPKD have an additional anatomical disruption of the medullary architecture induced by cyst formation, which will also diminish urinary concentrating capacity. Indeed, in a study including 177 ADPKD patients the observed renal concentrating defect paralleled the severity of anatomical changes caused by renal cysts.[34] Thus, according to this hypothesis, genetically determined progressive cyst formation will lead to higher vasopressin concentration. At the same time these higher vasopressin levels will lead to disease progression, as reasoned in the aforementioned paragraphs. These two processes are therefore expected to induce a vicious circle in ADPKD, predisposing to cyst growth, distortion of renal anatomy and renal function decline. This hypothesis may form an explanation why in subjects with ADPKD GFR remains relatively stable for a long period, while cyst formation progresses, then entering a phase with accelerated GFR decline.[25;53]
We acknowledge that this study has limitations. First, the cross-sectional observational design and the lack of data in non-cystic chronic kidney disease do not allow firm conclusions on the possible causal relationship between vasopressin and disease progression. Second, patients were

allowed to use their own medication, including diuretics. These drugs may influence plasma osmolarity and consequently copeptin concentration. However, adjustment for diuretic use in our multivariate models did not change our results. Third, we did not perform a water deprivation test, so are unable to draw firm conclusions whether ADPKD patients indeed do not respond effectively to vasopressin and have an impaired urinary concentrating capacity.
Strengths of our study are that, as far as we know, this is the first clinical study looking into the association between endogenous vasopressin and disease severity in ADPKD. It provides a rationale for the intervention studies with vasopressin receptor antagonists that are being conducted at this moment in subjects with this disease. Furthermore, we measured various indices of disease severity in ADPKD patients using the gold standards, being clearance of iothalamate for glomerular filtration rate, clearance of hippuran for effective renal blood flow, MRI for total renal volume and 24hr urinary excretion of albumin for albuminuria.

What may be the consequences of our findings? First, they may help shed light on the pathophysiology of ADPKD. Second, there may be clinical implications. At the moment, a large scale randomized clinical trial is being conducted to investigate efficacy of a vasopressin-2 receptor antagonist in ADPKD (TEMPO ¾ study, NCT00428948). This study is performed in patients with still relatively well preserved renal function (eGFR at baseline >60 mL/min), but large renal volume (total renal volume >750 ml). Our finding that patients with more severe ADPKD, as assessed by lower GFR, higher total renal volume and/or higher albuminuria, have higher copeptin levels suggests that patients with these characteristics may need higher dosages of a vasopressin receptor antagonist to effectively block the hormonal activity of the higher vasopressin levels. Although not studied yet, it is expected that tolvaptan treatment increases copeptin levels by feed-back mechanisms and that the increase in copeptin with tolvaptan will reflect adequacy of vasopressin suppression. If so, it is tempting to hypothesize that in patients with ADPKD the increase in copeptin due to tolvaptan could be used as a short-term marker for long-term therapy effectiveness of this drug with respect to renoprotection and that the level of copeptin could be used to determine the effective treatment dose of this drug .

In conclusion, our study shows that in ADPKD, copeptin, a surrogate for vasopressin, is associated with disease severity. This finding supports the results of animal models for ADPKD, in which vasopressin-2 antagonists resulted in renoprotection and offers a good prospect for clinical intervention studies that are currently being conducted with these agents.

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Part III

Chapter 7

Rationale and design of the TEMPO ¾ study, Tolvaptan Efficacy and Safety in Management of Autosomal Dominant Polycystic Kidney Disease and its Outcomes.

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Abstract
Current clinical management of ADPKD is mainly focused on managing complications of the disease, not on therapeutic targeting of cyst development or prevention of renal failure. Recently, tolvaptan, a selective vasopressin V2 receptor antagonist, was proven to inhibit renal cyst growth and preserve renal function in animal models of polycystic kidney disease. The TEMPO ¾ study is the first phase 3 clinical trial to examine the long-term effectiveness and safety of tolvaptan in patients with ADPKD.
This multicenter, double-blind, placebo controlled trial includes patients with ADPKD who have a relatively preserved renal function (baseline eCrCl ≥ 60 mL/min) and are anticipated to have progressive renal disease (age ≤50 years and total kidney volume (TKV) measured with MRI ≥750 mL). Primary outcome is rate of TKV percent change from baseline for tolvaptan relative to placebo. Secondary outcome parameters include time to multiple ADPKD progression events, among others time to a 33% increase in serum creatinine.
From March 2007 through January 2009, 1445 ADPKD subjects were enrolled. Preliminary baseline median TKV was 1.46 L and eCrCl was 105 ± 34 mL/min. A pre-specified, blinded, sample size re-calculation at 2/3 enrollment confirmed the likely power of the study to detect 20% differences from placebo in the primary and key secondary endpoints at $p<0.05$.
Results from this randomized clinical trial will show whether treatment with a vasopressin 2 receptor antagonist effectively inhibits cyst growth and disease progression in ADPKD patients.
Trial registration: NCT00428948

Introduction

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is the most common renal hereditary disease (caused in most cases by a mutation in the PKD1 or PKD2 gene[1;2]) . The disease is a systemic disorder characterised by progressive cyst formation in both kidneys, with progressive renal enlargement often leading to end stage renal disease. Other renal symptoms include pain, hypertension, gross haematuria, nephrolithiasis and mild albuminuria. Current therapies are directed towards limiting morbidity and mortality from complications of ADPKD,[3] but not specifically targeting the inhibition of cyst formation.
Although renal failure is the most feared consequence of the disease, measurement of glomerular filtration rate (GFR) is a poor marker of disease severity and progression in early phases of the disease. GFR remains intact during a prolonged period (typically decades) through compensatory hyperfiltration, but ultimately declines sharply thereafter. Gradual anatomical distortion, however, likely parallels the actual loss of functioning glomeruli.[4] The Consortium for Radiologic Imaging Studies of Polycystic kidney disease (CRISP) showed that total kidney volume at entry into the study predicted future renal function deterioration.[5] Therefore, in early ADPKD, total kidney volume (TKV) is likely to be a more sensitive marker of disease progression than kidney function.
Improved knowledge of genetic, molecular, and cellular mechanisms underlying cyst formation in ADPKD has resulted in the discovery of potentially effective therapeutic targets.[6] Vasopressin, acting on Vasopressin 2 (V2) receptors, increases intracellular cAMP in distal nephron segments including the collecting ducts, promoting chloride driven fluid secretion apically. Cyclic AMP also stimulates B-RAF/MEK/extracellular signal-regulated signalling, mitogenesis and proliferation of polycystic kidney epithelial cells or of wild-type kidney epithelial cells under experimental conditions of calcium deprivation.[7;8] Localization of the V2 receptors in the distal nephron and collecting duct,[9] corresponds to the main site of cystogenesis in Autosomal Recessive Polycystic Kidney Disease (ARPKD) and arguably in ADPKD,10 and increased circulating levels of vasopressin in animal models[11;12] and in patients with ADPKD[13;14] provided the rationale for experimental studies with vasopressin V2 receptor antagonists. The vasopressin V2 receptor antagonist OPC-31260 indeed inhibited cyst formation in animal models for ARPKD, nephronophthisis 11 and ADPKD.[12] Tolvaptan, another vasopressin V2 receptor antagonist, with high potency and selectivity for human vasopressin V2 receptor, proved effective in a rat model for ARPKD.[15] Moreover, genetic elimination of arginine vasopressin (AVP) in this model yielded animals born with normal kidneys that remained relative free of cysts unless an exogenous V2R agonist was administered.[16]
Because tolvaptan (Samsca®) induces free water clearance, it is approved by the US Food and Drug Administration (FDA) for "the treatment of clinically significant hypervolemic and euvolemic hyponatremia (serum sodium <125 mEq/L or less marked hyponatremia that is symptomatic and has resisted correction with fluid restriction), including patients with heart failure, cirrhosis, and the Syndrome of Inappropriate Antidiuretic Hormone (SIADH)" with "Important limitations: Patient requiring intervention to raise serum sodium urgently to prevent or to treat serious neurological symptoms should not be treated with SAMSCA" and "It has not been established that raising serum sodium with SAMSCA provides a symptomatic benefit to

patients" The European Medicines Agency (EMA) has also approved the drug for "Treatment of adult patients with hyponatremia secondary to the syndrome of inappropriate antidiuretic hormone secretion."These approvals were based on studies of efficacy and safety in hyponatremia[17] and safety in patients with congestive heart failure, with or without hyponatraemia.[18;19] Phase 2 studies in ADPKD patients showed that split-dose administration of tolvaptan was more effective than a single dose in achieving sustained suppression of vasopressin action, as evidenced by a 24 hour urine osmolality reduced below 300 mOsm/L.[20] A phase 2 open-label trial in 46 and 17 ADPKD patients investigating the long-term safety, tolerability and efficacy of split-dose regimens has completed 3 years of treatment and is ongoing in the US[21] and Japan[22] respectively. Based on promising results in animal models and early clinical studies with respect to efficacy and safety, we have designed and initiated the first large clinical trial to examine the effectiveness (rate of TKV change and time to multiple ADPKD progression events) of a selective vasopressin V2 receptor antagonist (tolvaptan) in young patients at relatively early stages of ADPKD.

Materials and Methods

Study population

ADPKD patients (diagnosis based upon Ravine criteria[23]) between 18 and 50 years were eligible for study-participation. Patients furthermore had an estimated creatinine clearance by the Cockcroft-Gault equation[24] (eCrCl) above 60 mL/min and a measured TKV (sum of right and left kidney volumes) by magnetic resonance imaging (MRI) larger than 750 mL. Detailed study inclusion and exclusion criteria are presented in Table 1.

Study design and setting

The TEMPO ¾ study was designed as a multi-center, double-blind, placebo-controlled, parallel-arm trial in subjects with ADPKD. Subjects were enrolled worldwide (North and South America, Europe, Japan, Russia and Australia). After determining eligibility (Table 1), patients were randomized with stratification to one of the two treatment groups (2:1 ratio, tolvaptan: placebo). Stratification factors include baseline hypertension (present or absent), eCrCl (≥ 80 mL/min or <80 mL/min), and TKV (≥1000 mL or <1000 mL).) Hypertension is defined as a systolic blood pressure >139 and/ or diastolic blood pressure >89 mm Hg or use of antihypertensive treatment.

Figure 1 schematically represents the trial design. Three split-dose regimens of tolvaptan and matching placebo are tested: low (45/15 mg), medium (60/30 mg) and high (90/30 mg). Subjects begin treatment with the lowest dose and after each 1-week safety assessment, are titrated to the next higher dose treatment group, until a level of intolerability or the highest dose of treatment is reached. After the titration phase, participating subjects remain on the highest tolerable dose until 36 months of treatment is completed. Patients are evaluated every 4 months during treatment and twice for 2-6 weeks after treatment concludes.

Table 1: Eligibility criteria of the TEMPO ¾ study

Inclusion Criteria:
• Adult subjects providing informed consent [Defined as men or women ≥18 years and ≥ regional legal age of maturity to age 50 years.]
• Adult subjects with a diagnosis of ADPKD
• Willingness to comply with reproductive precautions Women who are capable of becoming pregnant must be willing to comply with approved birth control from two-weeks prior to, and for 60 days after taking investigational product.
• Estmated creatinine clearance (eCrCl) ≥60 mL/ min within -31 days of randomization [Established using Cockcroft Gault.[33]]
• Rapid estimated rate of kidney volume increase based on total kidney volume ≥750 mL by MRI at randomization [Excluding those meeting volumetric criterion solely due to six or fewer predominant cysts.)]
Exclusion Criteria:
• Subjects who, in the opinion of the study investigator or sponsor may present a safety risk
• Subjects who are unlikely to adequately comply with the trial's procedures [Due to medical conditions likely to require an extended interruption or discontinuation, history of substance abuse or non-compliance)
• Subjects having contraindications to, or interference with MRI assessments
• Subjects taking medications or having concomitant illnesses likely to confound endpoint assessments
• Subjects taking other experimental (i.e., non marketed) therapies, or taking approved therapies for the purpose of affecting ADPKD cysts, or those taking or having a history of taking tolvaptan

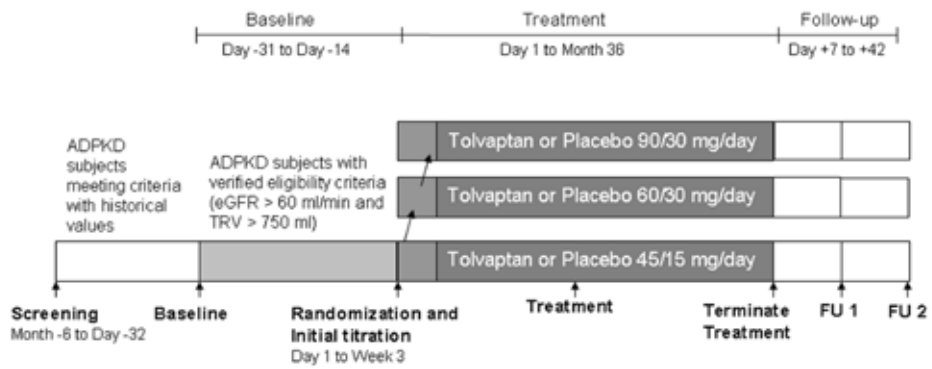

Figure 1: schematic trial design of the TEMPO ¾ study

Trial treatments

Tolvaptan or placebo tablets are given orally, twice daily for three years. Dosing occurs on waking and approximately 9 hours later, irrespective of meals. Exact timing is adjusted based on wake/sleep habits. However, dosing times are consistent for each individual's daily dose to avoid breakthrough. After titration to tolerability, the maintenance phase starts, during which subjects may down-titrate at any time. Subjects unable to tolerate the lowest dose are discontinued from investigational product use, yet are asked to continue participation in study exploratory ADPKD outcomes assessment (Table 2) in the trial. Subject compliance is monitored by pill counts as drug is returned. Non-compliant subjects (defined as discontinuation of investigational product for 30 consecutive days or >30% missed dosages intended for a period, without investigator and medical monitor approval) are deemed non-compliant and withdrawn from the trial.

Safety of subjects and optimal testing of tolvaptan efficacy requires standardization of background clinical care. Therefore, this trial includes a requirement for antihypertensive therapy in subjects with hypertension (SBP > 139 mm Hg and/or DBP > 89 mm Hg). Since deterioration of renal function in ADPKD is likely to be accelerated by hypertension, treatment is also required (unless otherwise contraindicated) in subjects with reproducible systolic blood pressure of 130-139 mm Hg and / or a diastolic blood pressure of 85-89. The protocol recommends first-line use of an angiotensin converting enzyme inhibitor (ACEi) or an angiotensin receptor blocker (ARB). Chronic use of diuretics is not allowed as they may impact certain assessments (as urine sodium or osmolality). In addition, recommendation for dietary restrictions (such as restrictions for salt, protein and caffeine) is left to the discretion of the principle investigator and therefore site specific. Additional site-specific or region-specific additions to the visit schedule have been allowed where requested by local regulatory authorities or ethics committees.

Subjects are given standard fluid ingestion recommendations, to help avoid dehydration and reflex vasopressin increases. They are asked to drink enough water to prevent thirst throughout the daytime period and an additional 1-2 cups of water before bedtime. During the titration phase, subjects were asked to self monitor changes in body weight, reporting changes of greater than 3% in body weight over any 7-day period to the trial physician.

Primary outcome

The primary outcome of the TEMPO ¾ trial is to determine the effect of tolvaptan on TKV in ADPKD. Primary efficacy endpoint is the rate of TKV change (normalized as percentage) for tolvaptan (combining all doses) relative to placebo.

Secondary outcomes

Secondary outcomes involve a composite secondary efficacy endpoint and non-composite secondary efficacy endpoints. Additional safety, pharmacokinetic-, pharmacodynamic and exploratory endpoints have been formulated.

The composite secondary efficacy endpoint is the time to multiple investigator-reported ADPKD clinical progression events (changes in blood pressure category, or the onset or worsening of hypertension requiring adjustment to hypertensive treatment), severe renal pain (requiring medical intervention), worsening albuminuria (by category), worsening renal function (33%

Table 2: Primary and secondary endpoints of the TEMPO ¾ study

Primary outcome endpoint:
Primary efficacy endpoint: Rate of total renal volume change (normalized as percentage) for tolvaptan (combining all doses) relative to placebo.
Secondary outcome endpoints:
Composite secondary efficacy endpoint: Time to investigator-reported multiple ADPKD clinical progression events (i.e. onset or progression of hypertension (need for hypertensive treatment), severe renal pain (requiring medical intervention), worsening albuminuria (by category), worsening renal function (25% change in reciprocal serum creatinine) for tolvaptan (combining all doses) relative to placebo while on treatment. Non-composite secondary efficacy endpoints: For tolvaptan compared to placebo: 1. Rate of eGFR change from post-dose baseline (end of titration) to last on-drug trial visit (using 1/serum creatinine as primary measure). 2. For subjects who are non-hypertensive at baseline, change from baseline for resting mean arterial pressure at scheduled clinic visits up to point of exposure to anti-hypertensive therapy for any reason 3. Change from baseline in kidney pain as assessed by 0-10 pain scale as average area under the concentration-time curve between baseline and last trial visit or last visit prior to initiating medical or surgical therapy for pain. 4. For subjects who are non-hypertensive at baseline, time to progress to any of: a) high-pre-hypertension (SBP >129 and/or DBP >84 mm Hg) b) hypertension (SBP>139 and/or DBP > 89 mm Hg) c) requiring anti-hypertensive therapy 5. For subjects who are taking anti-hypertensive therapy at baseline, percentage with clinically sustained decreases of blood pressure leading to a sustained reduction in antihypertensive therapy compared to baseline (while taking investigational product) at visits months 12, 24 and 36.
Safety endpoints:
Safety endpoints to be analyzed will include a descriptive summary of: 1. Reported adverse events 2. Vital signs 3. Clinical laboratory tests 4. ECG data
Pharmacokinetic endpoint:
1. Determination of tolvaptan and metabolite plasma concentrations
Pharmacodynamic endpoints:
For tolvaptan compared to placebo: 1. For urine, through spot osmolality and MCP1/creatinine ratios 2. For blood, cystatin C and BUN concentrations
Exploratory endpoint:
1. Fasting urine osmolality (at randomization and follow-up visit #2 only) 2. ADPKD outcomes and medical resource utilization. Analysis of additional events attributed to ADPKD for tolvaptan-treated subjects as compared to placebo, including their health-economic outcomes

increase in serum creatinine) for tolvaptan (combining all doses) relative to placebo while on treatment. A detailed description of those secondary endpoints is depicted in Table 2. An independent clinical events committee will adjudicate events contributing to the secondary composite endpoint for sensitivity analysis.

Data collection

Data as outlined in Table 3 is obtained. Patients are seen every 4 months. During each visit, medical history, concomitant medication, adverse events and tolerability are assessed. Blood is drawn and a spot urine sample is obtained to assess safety endpoints. In addition, a pregnancy test is performed (for women with childbearing potential). Subjects return used packages and receive new blinded study drug. Subjects are asked about investigational product tolerability and pain attributed to their kidneys (on a 0-10 scale).

Annually, a renal magnetic resonance imaging (MRI), using standardized procedures (see below) is obtained. An electrocardiogram (ECG) is performed at the beginning (baseline and end of titration) and end of the study (month 36 or early termination and second follow up visit). All blood and urine chemistry, ECG and MRI endpoint data are analyzed and read centrally. Data on PKD outcome assessments are collected through use of standardized case report forms and entered into a central database. Monitoring and auditing of the participating centers takes place to ensure data are correctly obtained. After 36 months (or an early termination visit), two follow-up visits are planned, one after 7-21 days and one 7-21 days after the previous visit. These visits are performed to assess potential persistence or rebound of effects as well as safety of the subjects upon study treatment withdrawal.

Table 3: Ongoing data collection of the TEMPO ¾ study

Assessment	*Number of times performed*	*Visit*
• MRI	4	Baseline, M12, 24, 36/ET
• ADPKD outcomes: composite endpoint components, in-clinic blood pressure, serum creatinine, albumin, renal pain score, origin of renal pain via exam or history, review of disease burden and health care utilization attributable to ADPKD	12	All visits
• Tolerability	12	All visits
• Pharmacokinetics (determination of tolvaptan and metabolite (DM-4103 and DM-4107) plasma concentrations	5	Baseline, week 3, M12,24, 36/ET
• Lab (hematology, serum chemistry, urinalysis and urine pregnancy)	12	All visits
• Physical exam	2*	Baseline and M36/ET*
• ECG	3	Screening, M36/ET and FU 2

Abbreviations are: MRI, magnetic resonance imaging; ADPKD, autosomal dominant polycystic kidney disease; ECG, electrocardiogram; M, month; ET, early termination; FU, follow up.

* for all other visits, a directed exam should be conducted at the investigators discretion if deemed necessary to assess changes in medical history, adverse events or other medically indicated parameters.

MRI for determination of kidney volume

Patients undergo a standardized abdominal MRI protocol without use of intravenous contrast, at baseline, month 12, 24 and 36. In case of early termination, MRI is obtained when the last MRI was more than 6 months ago. MRI acquisition protocol includes T2-weighted single-shot fast spin-echo (SSFSE/HASTE) images with fat saturation and three-dimensional spoiled gradient interpolated T1-weighted images without fat saturation. At some sites, T2/T1 weighted fast imaging (true FISP/FIESTA) series are added to improve the delineation of kidney borders. All MR images are acquired in the coronal plane at 4 mm slice thickness covering the entire kidneys during breath-hold(s).

MRI images are sent to the central reading facility for quality control and measurement of renal volume using T1 and T2 weighted MR images as defined in a study specific imaging charter. Alice™ software (Perceptive Informatics) is used to measure TKV by calculating the volume of serial renal outlines which have been verified by independent radiologists familiar with ADPKD. To maintain objectivity in the evaluations, the radiology reviewers will be blinded to patient name and sequence of acquisition. Any investigator site identifiers, assessments, and determinations will also be masked.

Estimation of power and sample size

In a large cohort of ADPKD patients, mean annual total kidney volume growth rate was approximately 5%5. Growth rate in the TEMPO ¾ study is expected to be higher since only subjects with large kidneys (baseline TKV ≥750 mL) are included. Assuming an average untreated growth rate of 7%, an average 20% reduction in growth rate deemed clinically significant, 85% power to detect this difference and a two-sided alpha of 0.045, then approximately 504 subjects (split 2 tolvaptan : 1 placebo) are needed. This sample size calculation uses the sample size formula for longitudinal trial provided by J. Lefante[25] and assumes (in 10 log scale) the total noise standard deviation and the standard deviation of the slope across subjects to be 0.017 and 0.0184, respectively. Taking into consideration a possible 20% withdrawal rate for the trial, about 600 subjects need to be enrolled in the trial. By doubling this, an enrolment of 1,200-1,500 subjects was targeted so the study is powered to provide a higher than usual degree of statistical significance for the primary endpoint and a reasonable ability to evaluate the secondary composite endpoint.

Statistical analyses

Analysis will take place after completion of the study. The analysis of the primary endpoint is to fit the log10 transformed TKV data to a linear mixed-effect Laird-Ware model, with a p-value derived by applying the 'sandwich' estimator of the covariance matrix to the Wald test of the treatment-time interaction of the model, using observed cases of all intent to treat subjects.
In addition to the primary analysis provided above, mixed model repeated measures (MMRM) analysis will be applied to the repeated measures of change from baseline in TKV (based on logarithm transformed data) as a sensitivity analysis. Mean difference of the two treatment groups at year 3 under the MMRM will be used to estimate the treatment effect at year 3. The MMRM includes stratification factors (hypertensive status, kidney volume status and creatinine clearance status at baseline and geographic region), visit, treatment, and treatment visit interaction as class variables and baseline TKV as covariate. Observed cases dataset will be used in this MMRM analysis.

Secondary composite efficacy will be analyzed using the Andersen-Gill approach of the extended Cox model for analysis of time to multiple events with p-value provided by the robust Wald test using 'sandwich' estimate for the covariance matrix[26;27]. No interim analysis will be performed for this study. The independent data monitoring committee will monitor the study safety as well as efficacy.

Ethical considerations

Institutional review boards / independent ethics committees approved the protocol and informed consent forms (ICF) in all participating centers according to regional requirements. The trial is conducted according to the International Conference of Harmonization (ICH) Good Clinical Practice Guidelines and all other applicable regulatory requirements and adheres to the ethical principles that have their origin in the Declaration of Helsinki. Subject privacy is ensured by de-identifying all submitted data and using a subject identification code.

All subjects have the right to withdraw from the study at any time during the trial. Safety of trial subjects is monitored by an unblinded independent data monitoring committee (IDMC).

Study Organization

The design and conduct of the study are overseen by a Steering Committee. An independent data monitoring committee (IDMC) has been established to monitor the safety and efficacy of the trial. The IDMC is managed by an independent statistical data analysis center. A Clinical Events Committee (CEC) was formed to independently adjudicate events contributing to the secondary composite endpoint for inclusion in a sensitivity analysis. All study committees are guided by charters defining their roles and responsibilities and methods specific to the committee.

Results

This study has been submitted to clinicaltrials.gov. Identifier of this trial is NCT00428948.
Included are 1445 adult men and women (aged 18-50 years) in a relative early stage of ADPKD. Subjects were screened and enrolled at 132 sites worldwide; 38 sites in the Americas, 56 sites in Europe, 30 in Japan and 8 sites in Australia. Recruitment started in January 2007 and closed in January 2009. The final patient is expected to complete the trial in February 2012.
Preliminary baseline characteristics of the study population are described in Table 4 for the group as a whole and for women and men separately. These preliminary data approximate the final results for the study, but are still in the process of quality-control auditing. In total, 1445 subjects are randomized in the TEMPO ¾ study (52% men, aged 39±7 years). Median TKV is almost 1.5 L, approximately twice the minimum TKV needed to meet the inclusion criteria. Estimated kidney function is on average relatively well preserved at baseline (eCrCl 105 mL/min), with approximately 30% of subjects having an eCrCl between 60 and 80 mL/min. More then 75% of the subjects use at least 1 antihypertensive drug to control their blood pressure. Median urinary albumin/creatinine ratio is 3 mg/mmol for the whole group, over 50% subjects having micro-albuminuria (cut-off values defined as 2.8-28 mg/mmol for women and 2.0-20 mg/mmol for men) and 5% having overt proteinuria (>28 or 20 mg/mmol, respectively). Medication interfering with the renin-angiotensin-aldosterone system is used by nearly half of the subjects. The percentage of men treated with antihypertensive drugs is slightly higher than in women. Men furthermore have larger kidneys and a significantly higher eCrCl (all $p<0.001$), although the eGFR (MDRD) is approximately the same, which is explained by a higher body surface area for men.

Table 4: Preliminary baseline characteristics of participating subjects in the TEMPO ¾ study

	All (n=1445)	*Women (n=699)*	*Men (n=746)*
Age (yr)	39± 7.1	39±7.1	387.1
Body mass index (kg/m^2)	26±5.1	26±5.5	27±4.6
Systolic blood pressure	126±14	126±13	131±14
Diastolic blood pressure	82±10	81±9	84±10
MAP (mm Hg)	98±11	96±10	99±11
Subjects taking ≥ 1 antihypertensive, n (%)	1108 (77)	495 (71)	613 (82)
Subjects taking ACEi and / or ARB, n(%)	696 (48)	313 (45)	383 (51)
Total kidney volume (l)	1.46 (1.07-2.01)	1.30 (0.98-1.73)	1.64 (1.19-2.23)
Serum creatinine (mg/dl)	1.0±0.3	0.9±0.2	1.2±0.3
Urinary osmolarity (mOsm/l)	502±179	477±177	525±177
Urinary ACR (mg/mmol)	3.2 (1.7-7.0)	3.5 (2.0-7.1)	2.8 (1.5-6.7)
Gross proteinuria, n(%)	75 (5.5)	50 (7.0)	25 (3.8)
eGFR (MDRD, ml/min/1.73 m^2)	79±23	79±21	78±24
eCrCl (Cockcroft Gault, mL/min)	105±34	99±31	110±35

Values are depicted as mean± standard deviation or median (25th-75th percentile) in case of skewed distribution. Gross proteinuria is defined as an ACR>28 mg/mmol for women and >20 mg/mmol for men. Abbreviations are: ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; ACR, albumin creatinine ratio; eCrCl estimated creatinine clearance.

A blinded sample size re-estimation was pre-specified in the protocol to be conducted either after 1000 subjects had been enrolled or at least 200 subjects have completed their 12 month visit, which ever came first. The blinded sample size re-estimation was conducted on Oct. 20, 2008, when more than 1000 subjects had been enrolled. The sample size re-estimation suggested that a sample size of 1400 subjects would be expected to provide at least 80% power to the key composite secondary endpoint of the study.

Discussion

The TEMPO ¾ study seeks to determine if tolvaptan inhibits TKV growth in patients with ADPKD and whether such changes might meaningfully impact the clinical course of the disease. The approach of study designers to achieving these goals followed these principles:

- The primary endpoint should focus on the established mechanism of action of tolvaptan by studying its effects on the cystic expansion as measured by **TKV.**
- Treatment effects can most readily be measured in a population where cystic kidneys are **rapidly growing.**
- Secondary endpoints will confirm the medical impact of these effects by measuring meaningful consequences of the disease i.e., **direct clinical outcomes**.
- If renal structure and function correlate with clinical outcomes, an intervention should begin while the kidney remains largely intact or **early in disease**.
- Outcomes must be expected to measurably worsen over a **reasonable observation period**.
- Where endpoints are particularly clinically important and extraneous influences can be measured or controlled; stratification and standardization across treatment groups can help **control confounders**.

The primary endpoint of the trial is change in TKV. The Consortium for Renal Imaging Studies in Polycystic Kidney Disease showed that kidney enlargement results from the expansion of cysts in patients with ADPKD. This growth is continuous and quantifiable (as shown by MRI) and is associated with decline of renal function. Higher rates of kidney enlargement are associated with a more rapid decrease in renal function.[4;5] In a large number of animal studies, treatments that inhibited renal enlargement, also protected renal function.4 Furthermore, several studies show that the rate of increase of renal volume can reliably be measured.[28-30] These data provide support for the assumption that a change in rate of renal volume growth is a valid endpoint to assess the efficacy of drugs in ameliorating rate of disease progression in ADPKD. Based on the principles outlined above, patients were selected if they had a relatively rapid course of disease, as evidenced by a large TKV with a relatively young age but with relatively preserved renal function. Nevertheless, as renal function is such an important clinical consideration, it is included both as a component of the composite secondary endpoint and as a separate endpoint defined as a rate of decline over time as compared to placebo/standard care.

The subjects included in this study are ADPKD subjects with an age ≤ 50 years, an eCrCl ≥60 mL/min and a TKV ≥ 750 mL. These criteria were formulated to include subjects at a relatively early stage of their disease, but with a high likelihood of disease progression. This was done for several reasons. First tolvaptan is believed likely to delay disease progression, not restore renal function. Second, given that ADPKD is a progressive condition, intervention as early in life as possible to delay or prevent long-term consequences (including renal failure) seems most appropriate. Third, young patients with high TKV are likely to have a more difficult course than patients of the same age with smaller kidneys, since larger kidneys appear to be associated with hypertension, pain, haematuria, albuminuria, and renal function decline.[5] Assessment of a treatment effect on the secondary outcomes as defined in the TEMPO ¾ study will therefore

be feasible. Fourth, these patients, who have at baseline a reasonably preserved renal function, but already a large renal volume, are likely to be rapid progressors and prone to develop renal failure.[5] This specific subgroup would benefit most from a potential effective treatment.
For the secondary endpoint analysis, two analyses will be performed. The first one includes all the events observed during the double blind treatment period starting from the date of first dose of study medication. The second one includes all the events observed during the double blind treatment period from week 3 / end of titration to the end of the double blind treatment period, using week 3 / end of titration as new baseline. This last analysis will be a sensitivity analysis. Rationale for this sensitivity analysis is that there may be instability of fluid-related variables during the protocol-mandated switch of concurrent hypertensive medication in the titration period. Furthermore, vasopressin antagonists may, due to their aquaretic effect, have reversible, short-term hemodynamic effects on systemic blood pressure, GFR and urinary albumin excretion.[31] Such short-term reversible effects may obscure the efficacy of these drugs on long-term disease outcome. By adopting week 3 / end of titration values as baseline for assessment of long-term efficacy this potential source of bias is circumvented.
Phase 1 and 2 studies showed that the time to reach peak concentration (Tmax) of tolvaptan ranges from 1 to 4 hours[32] and mean half-life of tolvaptan ranged from 3 to 20 hours (data on file). Subjects with ADPKD respond similarly to oral doses of tolvaptan as healthy subjects, including a decrease in urine osmolality and an increase in 24h urinary volume. Because successful treatment of ADPKD is presumed to require constant inhibition of the vasopressin V2 receptor, dose regimens have been selected to provide as constant and complete an inhibition as would likely be tolerated by individuals taking tolvaptan chronically. A twice-daily regimen is designed to produce a maximal inhibition during waking with a gradual fall-off effect during sleep. This dosing scheme has been chosen to prevent intolerable polyuria and nycturia. Urine osmolality has been used as a surrogate of vasopressin V2 receptor inhibition. Normally, urine osmolality only increases above plasma osmolality (approximately 290 mOsm/l) when vasopressin is acting at the kidney's distal collecting ducts. When spot urine osmolality remains below 300 mOsm, effective vasopressin V2 receptor inhibition can be assumed. To induce the highest tolerable efficacy, a titration strategy with split-dosing regimens of 45/15, 60/30 and 90/30 mg was chosen for the TEMPO ¾ study.

In conclusion, the TEMPO ¾ Study is the first clinical study investigating the efficacy of a selective vasopressin V2 receptor antagonist (tolvaptan) in ADPKD. Baseline data show that we were able to include a large number of ADPKD patients relatively early in their disease, with a high likelihood of disease progression, as suggested by their relatively young age, relatively preserved renal function, but large kidneys. A blinded sample size re-calculation performed after 1000 subjects were enrolled, confirms that the trial has appropriate power to address the primary and secondary outcomes of this study. We hypothesize that tolvaptan will be able to inhibit or reduce renal volume growth and improve clinical outcomes in these ADPKD patients and might be an effective therapeutic option for ADPKD patients in an early phase of the disease.

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Part III

Chapter 8

Vasopressin V2 receptor antagonism in a mouse model for Autosomal Dominant Polycystic Kidney Disease: Optimal timing and dosing of the drug.

Submitted

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Abstract

The renoprotective effect of a vasopressin V2 receptor antagonist (V2RA) is currently tested in a clinical trial in early ADPKD. If efficacious, this warrants life long treatment with V2RA, however with associated side effects as polydipsia and polyuria. We questioned whether we could modify the side effects without influencing the renoprotective effect by starting the treatment later in the disease, or by lowering drug dosage.

To investigate this, we administered the V2RA OPC-31260 at high (0.1%) and low (0.05%) dose to a tamoxifen-inducible kidney epithelium-specific Pkd1-deletion mouse model starting treatment at day 21 (early) or 42 (advanced). After 3 and 6 weeks of treatment, we monitored physiologic effects, e.g. water intake and potential renoprotective effects, e.g. kidney weight and cystogenesis.

Initiation of V2RA treatment at advanced stage of the disease lacked renoprotective effects and had less pronounced physiologic effects than early initiation. When started early, both after 3 and 6 weeks of treatment, the V2RA caused dose-dependent physiologic effects. On high dose, water intake at both time points was 2-fold higher than on low dose ($p<0.001$). After 3 weeks on high dose, cyst ratio and kidney weight were reduced vs. untreated controls (18 vs. 25%, $p=0.05$ and 0.33 vs. 0.45g, $p=0.03$, resp.). After 6 weeks of treatment however, no significant renoprotective effect was noted, even at a high dose (cyst ratio 24 vs. 27%, $p=0.12$ and kidney weight 0.55 vs. 0.66g, $p=0.38$). After 6 weeks of treatment, mice receiving high dose also had lower water intake than after 3 weeks (2-fold vs. 4-fold increase resp. compared with untreated controls, $p=0.001$).

Our results suggest that intervention with a V2RA should be instituted early in ADPKD, preferably at high dose, and that it might be necessary to either further increase the dosage of this drug later in the disease to reach renoprotection, or to switch to combinational treatment with other drugs.

Introduction

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is the most common renal hereditary disease, characterized by progressive cyst formation in both kidneys, often leading to end stage renal disease.[1,2] The majority of patients (85%) have a mutation in the PKD1 gene.[3,4] Current clinical management of ADPKD is mainly focused on managing complications of ADPKD, because as yet no proven renoprotective treatments exist that are able to inhibit cyst formation and thus to prevent renal failure.

Over the past 5 years, experimental studies have suggested a central role for vasopressin and 3'-5'-cyclic adenosine monophosphate (cAMP) in promoting cyst growth, kidney enlargement and renal function decline in ADPKD5-8. Inhibition of vasopressin by either a pharmacological agent such as a Vasopressin V2 receptor antagonist (V2RA)9-11 or by drinking more water[12] led to reduced growth of cysts and renal function preservation in animal models of polycystic kidney disease.

At the moment, a large scale, randomized controlled trial is being conducted with a V2RA at high dosage for ADPKD patients early in the disease course.[13] If proven efficacious, important questions will, however, remain after completion of this clinical study. First, it will not be known whether the V2RA will be effective in ADPKD patients when started later in the disease. Because ADPKD is a progressive condition,[14] it seems rational to start an intervention as early in the disease process as possible to delay or prevent long-term consequences as renal failure. Second, it will not be known whether low dosages of the V2RA will be renoprotective. When given to healthy, normally hydrated volunteers, or ADPKD patients, a V2RA causes an increase in urinary volume, which is dose dependent up to 4 times compared with placebo.[15] As a consequence polyuria, and especially nycturia causing disturbed night rest, may have a negative influence on quality of life. Given this side effect profile and the fact that potentially life long treatment is warranted, it could be an option to use lower dosages of V2RA. Third, since 24h urinary volume decreases in ADPKD patients after multiple doses of a V2RA,[16] the adverse effects may diminish after prolonged administration. Because of this, we were interested in the time course of the effects.

We therefore investigated the effect of different dosages of a V2RA in a Pkd1- deletion mouse model for ADPKD at different stages of the disease with 3 main study questions: first, whether treatment with a V2RA is equally effective when initiated later in the disease course as compared with early administration, second, whether lower dosages result in less polyuria and are equally effective in reducing cyst formation as higher dosages and third, whether V2RA effects change over time.

Materials and Methods

Experimental animals

In this study a tamoxifen-inducible, kidney epithelium-specific Pkd1-deletion mouse model is used. Upon administration of tamoxifen to these mice, a genomic fragment containing exons 2-11 of the Pkd1 gene is specifically deleted in renal epithelial cells and cysts are formed. The inducible Pkd1-deletion mice (tam-KspCad-CreERT2;Pkd1lox2-11/lox2-11) have been described previously.17 We administered tamoxifen (Sigma-Aldrich, St. Louis, MO, USA, 0.5 mg

dissolved in ethanol in 0.25 µl sunflower oil) for 3 consecutive days to mice at Post Natal day 11 (PN11) per gavage. This young age was chosen because most cysts will arise from the distal tubules as well as in collecting ducts when the gene is knocked out at an early age.[18] Tam-Ksp-Cad-CreERT2;Pkd1lox2-1l/lox2-11mice that received tamoxifen are indicated as iKsp-Pkd1del mice or ADPKD. Nine tam-KspCad-CreERT2;Pkd1lox2-1l/lox2-11mice did not receive tamoxifen and served as healthy controls (indicated as wild-types).

Study design

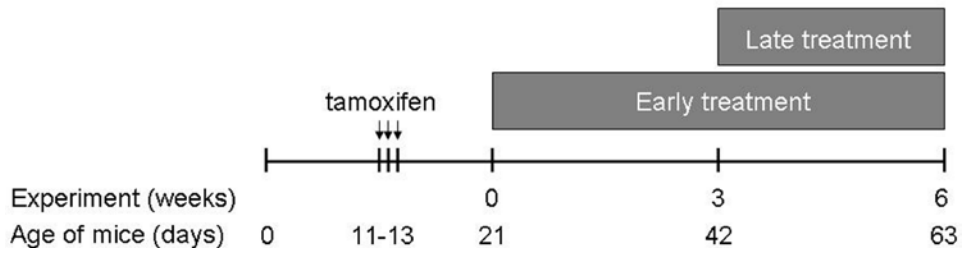
At 3 weeks of age, tamoxifen-treated male and female mice were divided into control groups and 3 treatment groups (Table 1). This time point serves as week 0 of the experiment. Figure 1 illustrates the time course of the experiment. The vasopressin V2 receptor antagonist OPC 31260 (V2RA, Otsuka Pharmaceutical Co, Tokushima, Japan) was added to ground rodent chow (AB Diets BV, RMH-B starch, 2103) at 0.05% (low dose, LD) and 0.1% (high dose, HD).

Table 1: Experimental groups. Early treatment was initiated at week 0, late treatment started at week 3 (see also Figure 1). Abbrevations are: V2RA, vasopressin V2 receptor antagonist; HD, high dose (0.1%); LD, low dose (0.05%).

	Groups	*n*	*Sacrificed at*		
			week 0	week 3	week 6
1	Wild-type	9	3	3	3
2	ADPKD, untreated	26	6	9	11
3	ADPKD, V2RA early, LD	15	.	.	15
4	ADPKD, V2RA early, HD	21	.	7	14
5	ADPKD, V2RA late, HD	10	.	.	10

Animals, water and food were weighed every week. Mice were housed with on average 4 mice per cage. Water intake and food intake was divided by the number of mice per cage to obtain an average intake per animal. V2RA intake per body weight was calculated by dividing the percentage in food intake by body weight of the mice. Animals were sacrificed at week 0, week 3 and week 6 of the experiment (Table 1 and Figure 1). All experiments were approved by the local animal experimental committees of the Leiden and Groningen University Medical Centers and by the Commission Biotechnology in Animals of the Dutch Ministry of Agriculture.

Figure 1: Study design



Experimental protocol

Twenty-four-hour urine outputs in individual metabolic cages were obtained before mice were sacrificed. The animals were weighed and anesthetized with isoflurane gas (0.5% isoflurane with a flow of 0.6 l/min). Blood was obtained by cardiac puncture for determination of plasma electrolytes, creatinine and urea. Both kidneys were removed and weight was measured on a precision scale. Half of the right kidney was placed into formaldehyde. The tissues were embedded in paraffin for histomorphometry and immunohistochemistry. The other half of the right kidney and the left kidney were frozen immediately in liquid nitrogen for mRNA isolation.

Plasma- and urine analysis

Creatinine, urea, potassium and sodium were measured in plasma and urine, using Kodak Ektachem dry chemistry (Eastman Kodak, Rochester, NY, USA). Copeptin was measured using a new sandwich immunoassay (B.R.A.H.M.S. AG, Hennigsdorf / Berlin, Germany), which was based on the assay described previously[19] To detect murine copeptin, the capture and detection antibodies were made in sheep and directed to amino acids 139-148 and 156 to 168 of proAVP. Urinary osmolarity was calculated as 2 * (Urinary sodium concentration +Urinary potassium concentration) + Urinary urea concentration.[20] Creatinine clearance in ml/min was calculated as (the urinary excretion of creatinine in 24h / plasma creatinine concentration) / 1440. Fractional urea excretion (FE Urea) was calculated as $((Plasma_{creatinine} * U_{urea})/ (Plasma_{urea} * U_{creatinine}))* 100$.

Immunohistochemistry and histomorphometric analysis

Transverse tissue sections (4 µm), including cortex, medulla and papilla, were stained for PAS and hematoxylin-eosin (HE) to measure total area of cysts,[9] and with Sirius red collagen stain to measure fibrosis.[21] The total area of the cysts and of fibrosis was quantified using Aperio Image Scope software (version 9.1.772.1570, Aperio Technologies Inc, Vista, CA, USA). Total area of cysts (corrected for tubular lumina) was divided by total area of the tissue and multiplied by 100% to obtain a cyst ratio, expressed as percentage. Total intensity of fibrosis staining was divided by total intensity on the slide (both positive and negative) and multiplied by 100% to obtain a percentage of fibrosis.

Tubular segment identity was characterized using antibodies against megalin[22] to identify proximal tubules, against Tamm Horsfall protein (uromodulin, Cappel-Organon Teknika, Durham, NC, USA) to identify the thick ascending limb of Henle and distal convoluted tubules, against Aquaporin-2 (Calbiochem La Jolla, CA) to identify principal cells of the late distal tubule and antibodies against Dolichos biflorus agglutinin (DBA, Sigma-Aldrich, Zwijndrecht, The Netherlands) to identify the collecting duct. Stainings were performed as described previously.[17;18;23]

mRNA expression analysis

Kidneys snap frozen in liquid nitrogen were stored at -80°C until further processing. Renal tissues were homogenized in PBS containing 1% 2-mercapto-ethanol using a MagNa Lyser Instrument (Roche). Total RNA was isolated from renal tissue homogenates using TRI-Reagent (Sigma-Aldrich) according to the manufacturers' protocol. cDNA was synthesized with Superscript III (Invitrogen). Quantitative gene expression analysis of Avpr2 and Aqp2 was performed on a Light Cycler 480 (Roche Applied Science) using 2x Sybr-Green master (ROX) (Roche Applied Science) according to manufacturers' protocol. Hprt (Hypoxanthine phosphoribosyl transferase) was used as a housekeeping gene. Primer sequences are available upon request. Data were analyzed according to the Pfaffl ΔΔCT method, taking PCR efficiencies into account.[24] Expression was calculated relative to the median of untreated iKsp-Pkd1del mice.

Statistical analyses

Analyses were performed with SPSS version 16.0 (SPSS Inc., Chicago, IL). Differences between the groups were tested using a non parametric independent sample test (Mann Whitney).

Results

Week 0

Non-treated ADPKD versus wild-type mice

At the start of the experiment (week 0), iKsp-Pkd1del mice were not different from wild-type mice regarding cyst ratio. Total renal weight (iKsp-Pkd1del mice 0.18g vs. wild-types 0.19g, p=0.7), plasma creatinine (13 µmol/l vs.15 µmol/l in wild-types, p=0.3) and urea (11 vs. 12 mmol/l, p=0.7) were also not different.

The iKsp-Pkd1del mice were divided into controls and different treatment groups (Table 1), i.e. a non-treated control group, a group treated with low dose V2RA early start, a group treated with high dose V2RA early start and a group treated with high dose V2RA late start. Formation of these groups and start of the experiment took place at day 21 after birth. Figure 1 depicts the time course of the experiment.

Body weight, food intake and water intake for the different groups throughout the 6 week experiment are presented in Figure 2. Wild-type mice had larger bodyweight and ate more compared with iKsp-Pkd1del mice. There were no differences between the different groups of iKsp-Pkd1del mice regarding body weight and food intake.

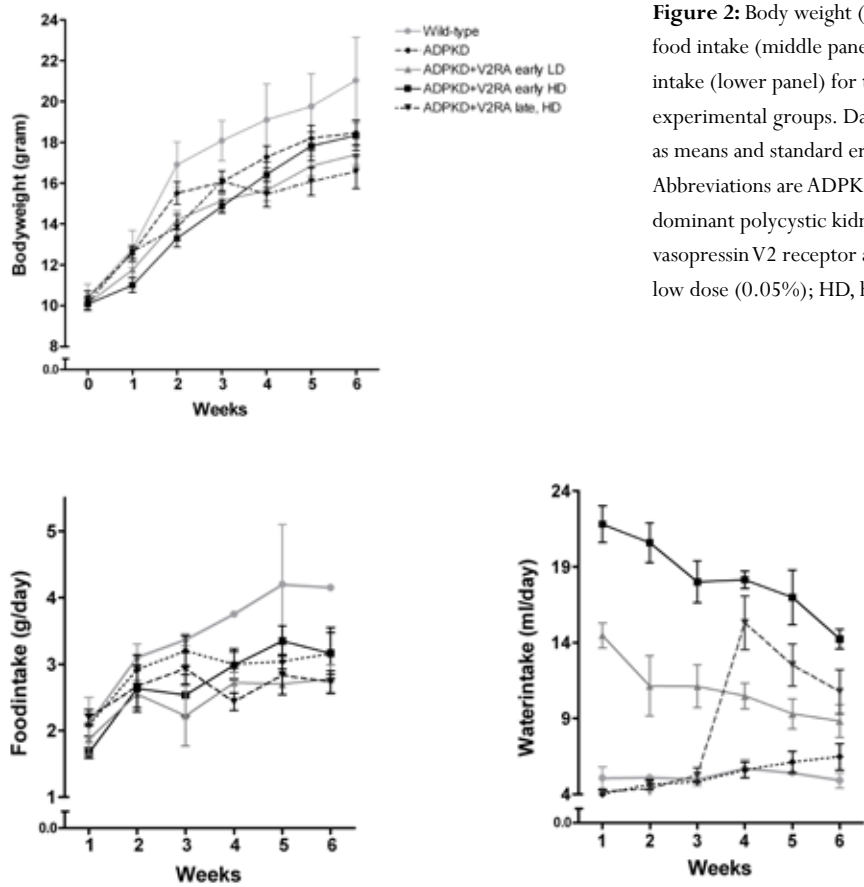


Figure 2: Body weight (upper panel), food intake (middle panel) and water intake (lower panel) for the different experimental groups. Data are presented as means and standard errors of the mean. Abbreviations are ADPKD, autosomal dominant polycystic kidney disease; V2RA vasopressin V2 receptor antagonist; LD, low dose (0.05%); HD, high dose (0.1%).

Week 3

Non-treated ADPKD versus wild-type mice

After 3 weeks of the experiment, iKsp-Pkd1del mice had higher 24h urinary volumes and lower 24h urinary osmolarity than wild-type mice (p=0.02), suggesting a decreased urinary concentrating capacity (Figure 3). IKsp-Pkd1del mice had significantly higher cyst ratios and larger kidneys than wild-type mice (p<0.001; Figure 5). Cysts were never megalin positive, suggesting that the cysts did not derive from proximal tubules (Figure 4). Most cysts were uromodulin positive; indicating the majority of cysts arose from distal parts of the nephron. Approximately 20% of the cysts, generally smaller cysts, were positive for aquaporin-2, indicating cysts derived from principal cells of the collecting ducts in a minority of cases. Sixty percent of the cysts were positive for lectin Dolichos biflorus agglutinin (DBA), identifying cysts derived from the collecting duct and late distal tubules. A large proportion of the cysts stained for uromodulin as well as DBA, which is in agreement with a previous published expression pattern for DBA and Uromodulin in mouse kidneys.[25] Creatinine clearance was not different between iKsp-Pkd1del mice and wild-type mice (150 vs.139 µl/min, p=0.6).

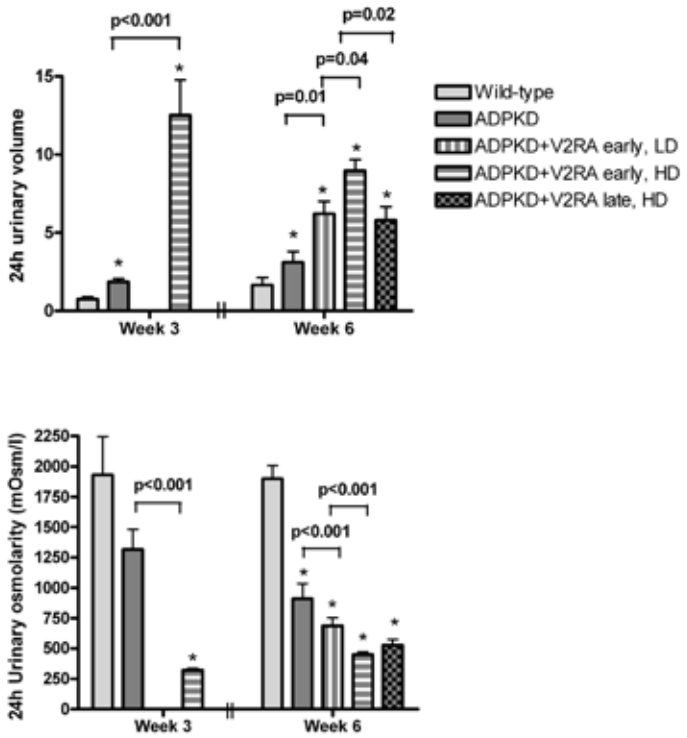


Figure 3: Physiologic effects of the vasopressin V2 receptor antagonist (V2RA) at week 3 and week 6 of the experiment: 24h urinary volume (upper panel), 24h urinary osmolarity (middle panel) and fractional urea excretion (lower panel) for experimental groups. Data are presented as means and standard errors of the mean. LD, low dose (0.05%); HD, high dose (0.1%);* indicates p<0.05 compared with wild-type.

Early start, high dose V2RA versus non-treated ADPKD

Treatment with the vasopressin V2 receptor antagonist (V2RA) at high dose induced more diuresis compared with the non-treated iKsp-Pkd1del mice, as shown by a higher 24h urinary volume (almost 7 times increased, p<0.001), a lower 24h urinary osmolarity (approximately 4 times decreased, p<0.001) and a higher fractional urea excretion (approximately 2 times increased, p<0.001, Figure 3). This was accompanied by increased water intake (p<0.001, Figure 2). After 3 weeks high dose V2RA treatment, cyst ratios (p=0.05) and kidney weight (p=0.03)

were significantly lower compared with the non-treated iKsp-Pkd1del mice (Figure 5). After treatment, cysts were still positive for uromodulin and aquaporin-2 and negative for megalin (Figure 4). There were no differences (p=0.49) in fibrosis between the treated (median1%) and the untreated iKsp-Pkd1del mice (median 0.7%). Creatinine clearance was not changed by V2RA administration. Median creatinine clearance was 119 µl/min in the treated animals and150 µl/min in the untreated animals (p=0.6).

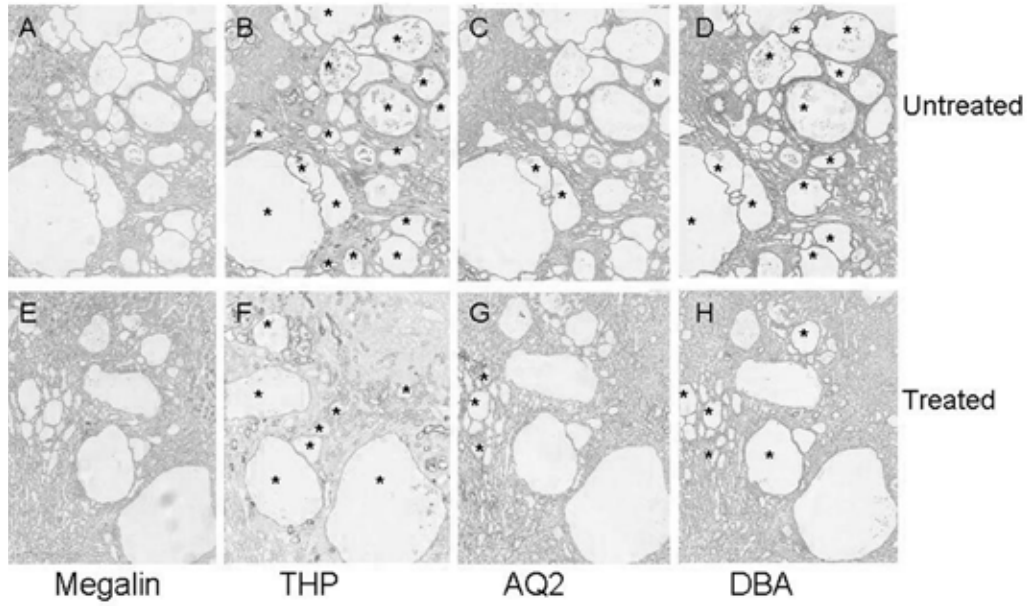
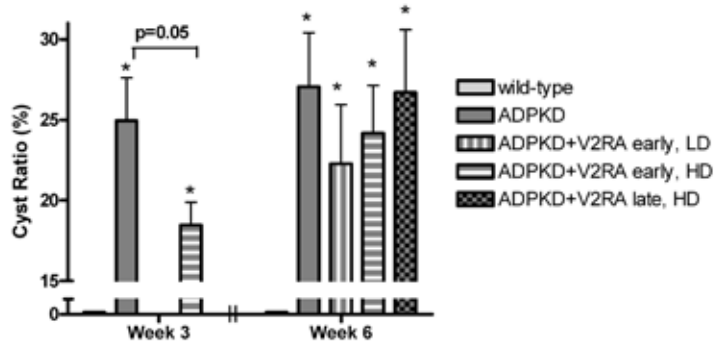


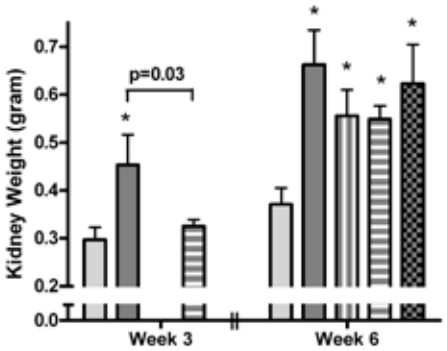
Figure 4: Tubular segment identity of cysts. Panels A-D show kidney sections from an untreated iKsp-Pkd1del mouse, sacrificed at 3 weeks. Panels E-H show kidney sections from a mouse treated with a high dose V2RA for 3 weeks. Sections are stained for the proximal marker Megalin (A&E), the distal markers Tamm Horsfall Protein (THP, B&F), Aquaporin-2 (AQ2, C&G), and lectin Dolichos biflorus agglutinin (DBA, D&H). * indicates positive staining of the cyst.

Week 6

Non-treated ADPKD versus wild-type mice

After 6 weeks of the experiment, untreated iKsp-Pkd1del mice had a higher 24h urinary volume and a lower 24h urinary osmolarity (p=0.05) compared with wild-types (Figure 3) suggesting a decreased concentrating capacity. Untreated iKsp-Pkd1del mice also had cysts (difference in cyst ratios p=0.007) and larger kidneys (p=0.03) than wild-types (Figure 5). Creatinine clearance was not different between iKsp-Pkd1del mice (141 µl/min) and wild-types (179 µl/min), p=0.37.





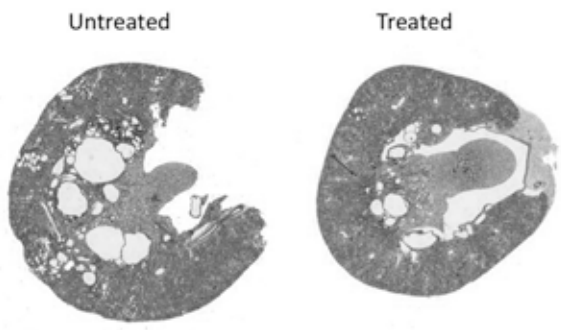


Figure 5: Renoprotective effects of the vasopressin V2 receptor antagonist (V2RA) at week 3 and week 6 of the experiment. Cyst ratio (upper panel) and kidney weight (middle panel) for experimental groups. Data are presented as means and standard errors of the mean. No statistically significant differences between the various ADPKD groups at week 6 were found. * indicates $p<0.05$ compared with wild-type. HD, high dose (0.1%). The lower panel shows representative kidney sections of an untreated iKsp-Pkd1del mouse and iKsp-Pkd1del mouse treated with high dose V2RA at week 3 of the experiment.

Early start, low dose versus early start high dose versus non-treated ADPKD mice

Treatment with the V2RA for 6 weeks resulted in larger 24h urinary volume ($p=0.04$) and lower 24h urinary osmolarity ($p=0.003$) with the higher dose inducing more effects than the lower dose (Figure 3). Fractional urea excretion was not different between the high and low dose group ($p=0.16$, Figure 3).

No significant renoprotective effects of the V2RA were observed at this time point. Although cyst ratios and kidney weights were slightly different between the treated and non-treated iKsp-Pkd1del mice the differences were not statistically significant ($p=0.4$ and $p=0.3$, resp. low-dose vs. untreated, Figure 5). Also the amount of fibrosis (median fibrosis on high dose was 3%, on low dose 5% vs. 6% in untreated animals) and creatinine clearance (median 192 µl/min on high dose, 246 µl/min on low dose and 141 µl/min in the group without treatment) were not different ($p=0.7$ and p-0.8, resp.) between the treated (either with high dosage or with low dosage of V2RA) and non-treated iKsp-Pkd1del mice.

Later start, high dose versus early start, high dose and non-treated ADPKD

Later initiation of treatment with high dose V2RA, started in week 3 of the experiment, induced less diuresis compared with early treatment with high dose V2RA ($p=0.01$, Figure 3). Although there was a trend towards a higher urinary osmolarity when treatment was initiated later in the disease course, this was not significant ($p=0.16$). There was no renoprotective effect of V2RA treatment when initiated later in the disease course: both cyst ratios and kidney weights were not different ($p=0.9$ and $p=0.8$, resp.) from non-treated iKsp-Pkd1del mice (Figure 5).

Comparison of high dose V2RA effects at week 3 and at week 6

Intake of the V2RA per gram body weight was not different for iKsp-Pkd1del mice receiving the high dose of V2RA after three weeks of treatment compared with iKsp-Pkd1del mice after six weeks of treatment (Figure 6). At week 3 as well as at week 6 of the experiment treatment with the V2RA resulted in significant physiologic effects (figure 6). Treated animals showed a higher water intake, lower urine osmolarity and a higher fractional urea excretion compared to untreated iKsp-Pkd1del mice. Mice at 6 weeks of treatment had less physiologic effects of the V2RA when compared to mice at 3 weeks of treatment: twenty-four hour urinary osmolarity was higher (321 vs. 450 mOsm/l, $p<0.001$), whereas water intake (14 ml vs.18 ml, $p=0.005$) and fractional urea excretion (39 vs. 67%, $p<0.001$) were lower (Figure 6).

Since the physiologic effects were lower after 6 compared to 3 weeks of treatment, we analysed serum levels of copeptin, a stable precursor of and a marker for endogenous vasopressin, as well as renal mRNA levels of the Vasopressin V2 receptor and aquaporin 2.

There was no difference in copeptin concentration between mice treated for 6 weeks when compared to mice treated for 3 weeks ($p=0.7$; Figure 6). Expression of both the vasopressin V2 receptor and aquaporin-2 was lower in mice after 6 weeks of treatment compared with mice at 3 weeks of treatment ($p=0.004$ and $p=0.006$ respectively).

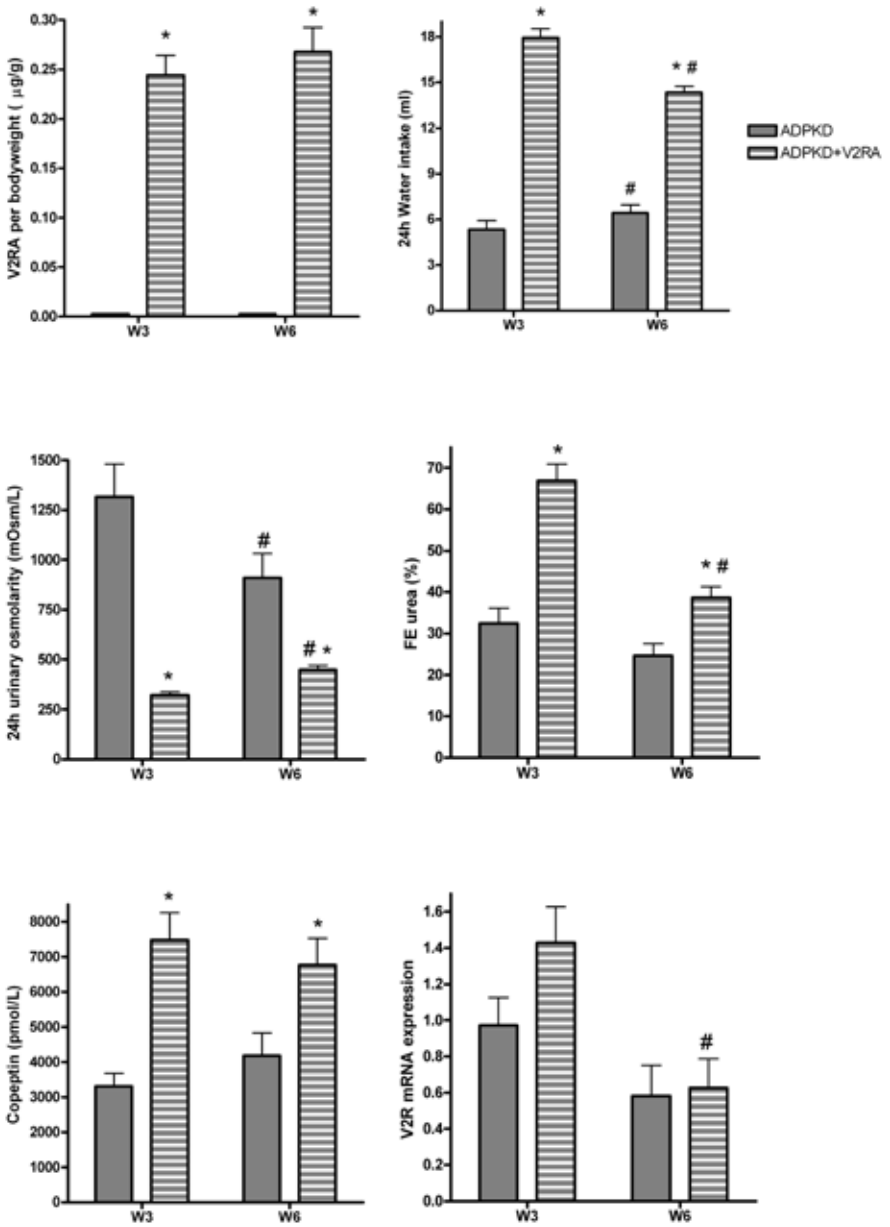


Figure 6: Comparison of V2RA intake (top left panel), physiologic effects of V2RA (24h water intake (upper right panel), 24h urinary osmolarity (middle left panel), and fractional urea excretion (middle right panel) and mRNA expression of the vasopressin V2 receptor (lower left panel) and of aquaporin-2 (lower right panel) between week 3 and week 6 of the experiment in untreated animals and in animals treated with high dose (0.1%) of the V2RA. * indicates $p<0.05$ for treated vs. untreated animals for that same time point. # indicates $p<0.05$ for week 6 compared to week 3 for that same experimental group.

Discussion

Vasopressin V2 receptor antagonist, when given at high dose early in the disease, reduced cyst formation and kidney weight in a Pkd1-deletion mouse model for ADPKD. When the same dosage was administered for a longer period of time, cyst ratio and kidney weight were lower, but this difference did not reach statistical significance anymore. The direct physiologic effects of vasopressin V2 receptor inhibition decreased after prolonged administration of the vasopressin V2 receptor antagonist (i.e. urinary osmolarity was higher and water intake was lower after prolonged administration compared with the earlier time point). Initiation of V2RA treatment at a more advanced stage in the disease had less pronounced physiologic effects compared with early start of treatment with the same dosage, and lacked renoprotective effects.

The beneficial effect of the vasopressin V2 receptor antagonist at week 3 of the experiment is in line with literature. Also in other models for polycystic kidney disease (autosomal recessive polycystic kidney disease, nephronophthisis and a Pkd2-model of ADPKD) this beneficial effect has been described.[9-11] Of note, the effects of vasopressin V2 receptor antagonists (V2RA) has, as far as we know, never been investigated in a Pkd1- model for ADPKD, although the majority of ADPKD patients (85%) have a mutation in the PKD1 gene.[3;26] Because of this high face validity with the human situation, the beneficial effect of the V2RA that we observed in this Pkd1-deletion mouse is of clinical relevance.

Aims of this study were to investigate whether we could modify the physiologic side effects without influencing the renoprotective effect of the V2RA by lowering drug dose or by starting treatment later in the disease. In normal physiology, vasopressin binds to the V2 receptors in the collecting ducts, increasing the water permeability along the entire collecting duct (through aquaporin-2), and increasing the urea permeability in the terminal inner medullary collecting duct.[27] Both processes result in water reabsorption, thereby reducing water excretion. After administration of the V2RA, water excretion (and consequently water intake) and fractional urea excretion indeed increased, whereas urinary osmolarity decreased. Of note, the observation that administration of a V2RA results in an increased fractional urea excretion, makes measurement of plasma urea as a measure of renoprotective effect of the V2RA less feasible. The observed physiologic effects of the medicament were dose-dependent. Lower dosage of V2RA resulted in less diuresis compared with the higher dosage in this animal model of ADPKD. This is consistent with the observed dose-dependent polyuria seen in healthy volunteers.[15] Initiation of V2RA treatment at high dose in a more advanced disease stage unexpectedly induced also less pronounced physiologic effects when compared with high dose treatment earlier initiated in the disease. Both lower dosage and initiation later in the disease could therefore partly overcome the physiologic side effects. In this study however, treatment with a low dosage as well as later initiation of treatment lacked renoprotection.

After 6 weeks of treatment the V2RA exerted physiologic and renoprotective effects when compared to untreated animals. However, when compared to 3 weeks of treatment these effects were diminished and did not reach statistical significance in all cases. The decreased efficacy

of the drug after prolonged administration could be caused by inadequate intake of the V2RA. Mice almost doubled in bodyweight throughout the experiment. However, study drug was administered as a percentage of food, and food intake also increased throughout the experiment. Consequently, the intake of the V2RA per gram bodyweight was not different at 6 weeks of treatment when compared with the earlier time point (Figure 6). This does, however, not exclude the possibility that there are differences in pharmacokinetic parameters of the V2RA at week 6 compared to week 3 of treatment. For instance, it could be that in these growing mice metabolism of the V2RA increases, thereby decreasing the serum concentration of the V2RA at the later time point, despite an equal intake of the drug. Unfortunately, the low blood volume obtained after sacrificing the animals did not allow to measure drug concentration. Another cause for the decreased efficacy of the V2RA could be the result of incomplete vasopressin V2 receptor antagonism. This could be caused by an increase in vasopressin levels or changes in vasopressin V2 receptor expression. Copeptin, the stable precursor of vasopressin,[28;29] was however not increased after prolonged administration. Vasopressin V2 receptor mRNA expression was decreased at 6 weeks of the experiment compared to the situation at 3 weeks. This means that there is more vasopressin per receptor present, which may lead to decreased efficacy of the V2RA and may explain the observation that physiologic effects decreased in the mice after prolonged administration of the drug. The phenomenon of decreased diuresis after multiple dosages of a V2RA is also observed in humans with ADPKD.[16] If this is indeed due to incomplete vasopressin V2 receptor blockade, this line of reasoning leads to the hypothesis that the dosage of vasopressin V2 receptor antagonist should be increased as the disease progresses to effectively block the hormonal activity of vasopressin.

An alternative explanation for the decreased sensitivity to V2RA after prolonged administration could be that vasopressin is effectively blocked, but other processes driving cyst formation become more important. It has been described that many signaling pathways are altered in cystic epithelial cells, directly or indirectly regulated by the PKD-proteins, and V2RA treatment may not block all of these pathways.2 In our model, approximately 20% of cysts are positive for aquaporin-2 (AQP2). These AQP2 positive cysts are the cysts that will be targeted by a vasopressin V2 receptor antagonist. Of note, the percentage of AQP2 positive cysts seems to be less than in the Pkd2 model that has been described in literature, where AQP2 expression is massively increased in the kidney.10 The majority of cysts in our model appears to derive from other tubular segments that most likely are not inhibited by the V2RA. In case this hypothesis is true, this might implicate that institution of combination therapy, targeting inhibition of cyst formation via various signaling pathways may overcome the decreased efficacy of the V2RA after prolonged administration. Examples of such medicaments could be somatostatin analogues[30-32] and mTOR inhibitors[33], although two recent clinical trials did not show a clear beneficial effect of mTOR inhibitors in ADPKD, both early[34] and later[35] in the disease.

In conclusion, the V2RA has beneficial effects on cyst growth in a Pkd1 deletion mouse model when initiated at high dose early in the disease on short term. Initiation of V2RA treatment at lower dose or at high dose later in the disease had less pronounced physiologic effects, but also lacked renoprotection. After prolonged administration, the V2RA induced lower cyst ratio and kidney weight compared to untreated animals, but this did not reach statistical significance. Physiologic side effects, though still significant, were also less pronounced at this later time point. This decreased responsiveness to the V2RA could be due to incomplete V2R antagonism or increased importance of other non-vasopressin related cystogenic pathways. Our results suggest that intervention with a V2RA should be instituted early in the disease course of ADPKD and at high dose, and that it might be necessary to either increase the dosage of this drug later in the disease to reach renoprotection, or to switch to combinational treatment with other drugs.

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Chapter 9

Summary and future perspectives

Summary

ADPKD is the most frequent hereditary kidney disease characterized by massive cyst formation in the kidneys, leading to end stage renal disease.[1] Presently it is not known whether clinical management of ADPKD is able to prevent or postpone renal failure. There is not much evidence to support dietary protein restriction,[2] strict blood pressure regulation[3] or treatment with angiotensin converting enzyme inhibitors[4,5] in prevention of renal function deterioration for these patients. It has to be noted though, that most of the studies with these regimens had too short follow-up, too small patient groups or included a control group that had almost no renal function deterioration to allow firm conclusions. The question whether these regimens really lack beneficial effect on renal function preservation remains therefore unanswered.

In the past decade, a better understanding of the pathogenesis of ADPKD[6] has led to potential new treatment options for this chronic condition.[7] Examples of these treatment options are triptolide,[8] roscovitine,[9] mammalian Target Of Rapamycin (mTOR) inhibitors,[10] somatostatin analogues[11-13] and vasopressin V2 receptor antagonists. A recent clinical trial showed that in early ADPKD, sirolimus did not slow kidney growth in 100 ADPKD patients, and there was a trend towards a better GFR.[14] In late ADPKD (433 ADPKD patients with a renal volume above 1500 ml at baseline) however, everolimus did not result in renal function preservation although it appeared to slow the increase of total kidney volume.[15] So far, a clear beneficial effect of mTOR inhibitors in ADPKD is therefore lacking. Regarding vasopressin V2 receptor antagonists, a selective vasopressin V2 receptor antagonist called tolvaptan was proven to inhibit renal cyst growth and preserve renal function in animal models of polycystic kidney disease.[16-18] This medicament is approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for treatment of hypervolemic and euvolemic hyponatraemia. These approvals were based on studies of efficacy and safety in patients with hyponatremia[19] and safety in patients with congestive heart failure, with or without hyponatraemia.[20;21]

This thesis focuses on the role of vasopressin in renal disease progression in ADPKD. It is divided into three parts. Part I describes our search for markers to assess disease severity in ADPKD. Part II investigates the potential detrimental role of vasopressin on these markers for disease severity in ADPKD, but also in other patient groups. Part III is a synthesis of the previous parts. This part contains two manuscripts on intervention with a vasopressin V2 receptor antagonist in ADPKD.

Part I: Measurement of renal damage in ADPKD

Because the clinical course of ADPKD is highly variable[22] (see also Figure 2 of the introduction), and all potentially effective experimental medicaments have side effects, exposing all ADPKD patients to these medicaments is not preferred. Ideally, treatment should be directed to patients at high risk for disease progression. To be able to identify such patients, there is a need for markers to assess disease severity. Part I describes our search for these markers. In nephrology, the most common way to assess disease severity is to measure glomerular filtration rate (GFR). In ADPKD, it has been hypothesized that measurement of glomerular filtration rate

is misleading in reporting the progression of ADPKD,[23] for GFR is believed to be stable in the beginning of the disease. This supposed stability of GFR, despite progression of renal anatomical abnormalities, is thought to occur because of compensatory hyperfiltration (see also Figure 3 of the introduction). GFR is thus assumed not to be representative of disease severity early in the disease.[23;24] However, clinical data supporting this hypothesis are lacking.

We therefore investigated renal parameters in ADPKD patients at different ages in comparison to healthy subjects (chapter 2). In this study, we found that already at young adult age, ADPKD patients have marked renal abnormalities, despite only modestly enlarged kidneys and a near normal glomerular filtration rate. These abnormalities include a renal hemodynamic profile characterized by decreased effective renal plasma flow and increased filtration fraction. This hemodynamic profile could indicate hyperfiltration. The phenomenon of hyperfiltration could explain why glomerular filtration rate stays stable for a number of years and then rapidly declines, as described in literature.[23;24] This suggests that glomerular filtration rate is indeed not an appropriate marker for disease severity. This finding emphasizes the importance of a search for better markers to assess disease severity. We found in these ADPKD patients that effective renal plasma flow, as well as total renal volume could be examples of these markers (chapter 2). These findings are in accordance with studies of the CRISP consortium,[25] showing that renal blood flow[26] and total renal volume[27] predict renal function decline.

Measurement of effective renal plasma flow and total renal volume are however time consuming and expensive. Because ADPKD is a disease characterized by structural abnormalities of renal tubuli, interstitial inflammation and fibrosis, we hypothesized that urinary biomarkers reflecting tubular damage and inflammation could be promising candidate markers to assess disease severity (chapter 3). Furthermore, urinary biomarkers are relatively easy to obtain and inexpensive to measure, making them favorable candidate markers. In ADPKD patients, a limited number of urinary biomarkers have been investigated so far, namely albuminuria, β2 microglobulin,[28;29] NGAL30 and Monocyte Chemotactic Protein-1 (MCP-1).[31;32] In general, these studies investigated only one biomarker and the promising results obtained for these markers have not been corroborated by others, except for albuminuria. Several studies showed that in ADPKD, albuminuria is associated with increased renal volume,[25;33] mean arterial blood pressure, filtration fraction,[33] renal growth and slope of glomerular filtration rate.[26] Albuminuria is therefore a valuable biomarker indicating disease severity and predicting outcome. In general, albuminuria is assumed to reflect predominantly glomerular endothelial damage. In ADPKD however, albuminuria might also reflect (inflammatory) tubular damage.

We therefore measured urinary biomarkers in ADPKD patients and in healthy controls and associated these markers with effective renal blood flow and total renal volume (chapter 3). We wanted to investigate whether these associations were independent of albuminuria for two reasons. First, albuminuria is already an established marker to assess disease severity in ADPKD.[33] The newly found urinary biomarker should therefore add to albuminuria with respect to assessment of disease severity. Second, such an analysis can provide insight into the issue whether

albuminuria in ADPKD is of glomerular- or tubular origin.

We found that urinary biomarkers from all segments of the nephron are elevated in ADPKD patients compared to healthy controls (chapter 3). The proximal tubular marker NGAL is associated with both effective renal blood flow, and total renal volume, independent of albuminuria. Other associations that are independent of albuminuria are the associations of the proximal tubular marker β2 microglobulin and the distal tubular marker H-FABP versus effective renal blood flow, and of the proximal tubular marker KIM-1 and the inflammatory marker MCP-1 versus total renal volume.

These findings are of interest for at least three reasons. First, we found a striking correlation between IgG and albuminuria, which could suggest albuminuria in ADPKD is predominantly glomerular in origin. Second, we found the distal tubular damage marker H-FABP not to be associated with total renal volume. This is in contrast with literature suggesting the main site of cystogenesis is distal in the tubule[34] (although the origin of cysts in early ADPKD remains a subject of debate[35]). Third, NGAL is the only proximal tubular marker that is associated with both effective renal blood flow and total renal volume in our study. An explanation for this phenomenon could be that NGAL reflects not only proximal tubular damage, but also inflammation and distal nephron damage, as is suggested in literature.[36] Based upon our study, we hypothesize that determination of urinary excretion of β2 microglobulin, H-FABP, KIM-1, MCP-1, but especially NGAL could have additional value in clinical practice to assess disease severity in ADPKD.

Part II: Vasopressin and renal damage severity

In this part, we investigated the potential detrimental role of vasopressin in causing renal damage in several patient groups. The antidiuretic hormone vasopressin is crucial for water regulation in the body. Its secretion is stimulated in response to an increase in plasma osmolarity or a decrease in plasma volume. Vasopressin mediates water reabsorption, thereby reducing water excretion,[37;38] diminishing 24h urinary volume and increasing urinary osmolarity. Despite its biological relevance however, vasopressin might in theory also contribute to chronic kidney disease progression by its effect on renal hemodynamics, blood pressure, and mesangial and / or epithelial cells.[39-41] Experimental studies showed that a sustained increase in vasopressin V2 receptor stimulation results in an increased renal plasma flow and particularly in an increased GFR.[42] This hyperfiltration might have deleterious consequences, in particular in diseased kidneys, resulting in renal hypertrophy,[43] proteinuria,[44] and accelerated renal function decline.[45-47] This proposed mechanism is supported by studies in rats with chronic renal failure, showing that increased water intake or chronic infusion of a vasopressin V2-receptor antagonist reduced proteinuria and lowered the incidence of glomerulosclerosis[45;48] and tubulo-interstitial fibrosis.[49] Similarly, chronic infusion of a V2-receptor antagonist reduced albuminuria and kidney weight in diabetic rats.[50]

Human data investigating the potential detrimental role of vasopressin on the kidney are, however, lacking. What prevented investigators to study potential associations between vasopressin

and renal damage could be that direct measurement of vasopressin is problematic. More than 90% of vasopressin in the circulation is bound to platelets, vasopressin is unstable in isolated plasma,[51] and most vasopressin assays have a relatively limited sensitivity. For this reason, we chose to measure copeptin, which is recently measurable using an enzyme-linked immunosorbent assay (ELISA). Copeptin is the C-terminal portion of the precursor of vasopressin that has been shown to be a reliable marker of vasopressin secretion and a useful substitute for circulating vasopressin levels.[52-54]

We first investigated, in a cross-sectional study, the association between vasopressin and albuminuria in a large general population cohort, including 7593 subjects. Albuminuria is important because it is a predictor of cardiovascular mortality and progressive renal function deterioration, not only in diabetics, but also in the general population.[55;56] It is assumed that urinary albumin excretion not only reflects glomerular damage, but is also related to systemic endothelial dysfunction. Treatments associated with lowering albuminuria result in a better renal[57] and cardiovascular[58] outcome. Identifying modifiable factors that cause a rise in urinary albumin excretion is therefore important, since intervention directed to these factors might be expected to result in a better renal and cardiovascular prognosis. In chapter 4, we show that in both males and females, a high concentration of copeptin is associated with a low 24h urinary volume and a high 24h urinary osmolarity, consistent with normal physiology. Furthermore, this study shows that copeptin is associated with urinary albumin excretion: the higher copeptin, the higher urinary albumin excretion, again, both in males and in females. This association remained significant after adjustment for age and potential confounders, among which medication that could potentially influence both vasopressin (and thus copeptin) secretion and urinary albumin excretion. The association between copeptin and urinary albumin excretion was found to be most pronounced in older subjects.

In an addendum to chapter 4, we elaborate on salt intake as a potential confounder for this association. Higher salt intake could induce a higher plasma osmolarity and thus more vasopressin release, and a higher sodium intake is also associated with more albuminuria, but by another mechanism. The addition of sodium intake to our models however, did not materially change the association that we found between copeptin and albuminuria. This observation makes it less likely that high salt intake is the common factor that induces an increase in albuminuria as well as in vasopressin, and supports the suggestion that vasopressin per se is detrimental. Although cross-sectional, the observed association between vasopressin and urinary albumin excretion is consistent with our hypothesis that vasopressin induces albuminuria.

Of note, we did not find an association between copeptin and renal function deterioration during follow-up in these healthy subjects. Maybe this is caused by the fact that healthy subjects are not susceptible enough to experience vasopressin's detrimental renal effects. We found that the association between copeptin and urinary albumin excretion was most pronounced in elderly, who probably already have some renal damage compared to the younger subjects. Also in experimental studies investigating the detrimental role of vasopressin, the animals under study

have some form of renal damage (as a 5/6 nephrectomy[45;46]) before experiencing these detrimental effects of vasopressin.

We therefore investigated in 548 renal transplant recipients, median 6.0 years after transplantation, the association between baseline copeptin and renal function decline during follow-up (chapter 5). In these renal transplant recipients, regulation and action of copeptin seemed to be consistent with normal physiology because we found, at baseline, a positive association between effective plasma osmolarity and copeptin concentration, and a negative association between copeptin and 24h urinary volume and fractional urea excretion. Moreover, we found a positive association between copeptin and urinary sodium concentration (as a surrogate for urinary osmolarity).
In addition, baseline copeptin was significantly associated with change in estimated glomerular filtration rate (eGFR; calculated using the 4-variable MDRD formula[59]) during follow up (median 3.2 years) in these patients. In multivariate regression analysis, the association of copeptin at baseline with change in eGFR during follow-up remained significant after adjustment for age, gender, baseline eGFR, proteinuria and other known risk factors for renal function deterioration, suggesting that a high concentration of vasopressin may cause renal function decline. This is in line with results from animal experiments. A chronic reduction in plasma vasopressin concentration slowed the progression of renal failure in Sprague-Dawley rats with impaired renal function,[45] whereas infusion of 1-Desamino 8-D-arginine Vasopressin (dDAVP) induced a rise in serum creatinine and in proteinuria in Brattleboro rats.[46]

In ADPKD, vasopressin has been hypothesized to play a role in cyst formation. Vasopressin promotes 3-5-cyclic adenosine monophosphate (cAMP) production by acting on vasopressin V2 receptors in the distal nephron and collecting ducts. This cAMP plays a major role in promoting cyst growth,[60] for it stimulates both chloride-driven fluid secretion and activation and proliferation of cyst-derived cells.[61] In line with this potential role of vasopressin in cyst formation in ADPKD are the findings in animal models of polycystic kidney disease, where blocking the effect of vasopressin (and consequently decreasing cAMP levels) by either a pharmacological agent16-18 or by drinking more water[62] led to reduction of cyst formation and renal function preservation. So far however, no studies have looked at the association between endogenous vasopressin levels and disease severity in humans with ADPKD.

We therefore investigated the association between copeptin and disease severity in 102 subjects with ADPKD (chapter 6). As markers of disease severity we studied effective renal blood flow, total renal volume and albuminuria. We found that in these ADPKD patients, plasma osmolarity was associated with copeptin concentration, suggesting a physiologic regulation of vasopressin. In contrast to the situation in subjects of the general population (chapter 4) and in subjects with a renal transplant (chapter 5), copeptin concentration was not associated with 24h urinary volume, 24h urinary osmolarity or fractional urea excretion. This suggests a lack of physiologic effects of vasopressin. Most importantly, we found that copeptin levels were indeed associated with disease severity: higher copeptin levels were associated with lower effective renal blood

flow, larger kidneys and more albuminuria. These associations were independent of age, gender, use of diuretics and GFR.

Part III: Vasopressin V2 receptor antagonists and prevention of progressive renal damage?
Our finding that copeptin is associated with disease severity in ADPKD supports the previously mentioned results of animal models for ADPKD, in which vasopressin-2 antagonists resulted in renoprotection16-18 and offers a good prospect for the large clinical intervention study that is currently being conducted with these agents. This study is called the TEMPO ¾ study (Tolvaptan Efficacy and Safety in Management of Autosomal Dominant Polycystic Kidney Disease and its Outcomes, phase 3 of 4). In chapter 7, the rationale and design of this trial are described and also preliminary baseline data are presented. This multicenter, double-blind, placebo controlled trial includes patients with ADPKD who have relatively preserved renal function (baseline estimated creatinine clearance ≥ 60 mL/min) and are anticipated to have progressive renal disease. Primary outcome is the difference in rate of percent change in total kidney volume for tolvaptan compared to placebo. Secondary outcome parameters include time to multiple ADPKD progression events, among others time to a 33% increase in serum creatinine and worsening albuminuria. From March 2007 through January 2009, 1445 ADPKD subjects were enrolled world wide. In our center, the University Medical Center Groningen, 52 patients participate in this trial, making the UMCG the second largest participating center. Preliminary baseline data show that this study includes a large number of ADPKD patients (746 males and 699 females, total 1445 patients) relatively early in their disease (estimated creatinine clearance is 105 ± 34 mL/min), with a high likelihood of disease progression, as suggested by their relatively young age (38.7± 7.1 years) and large kidneys (median total kidney volume for all patients is 1.5 L). A blinded sample size re-calculation performed after 1000 subjects were enrolled, confirms that the trial has appropriate power to address the question whether the vasopressin V2 receptor antagonist will be able to inhibit or reduce renal volume growth and improve clinical outcomes in these ADPKD patients and might be an effective therapeutic option for ADPKD patients in an early phase of the disease.

Although the effect on renal growth of a vasopressin V2 receptor antagonist is being studied in this randomized clinical trial at the moment, important questions will remain after completion of the TEMPO ¾ study. It will not be known whether the vasopressin V2 receptor antagonist will be effective in patients with lower eGFR. ADPKD is a progressive condition and probably the cysts that are formed early in life are the main contributor to eventual total cyst volume.[63] It is therefore questionable whether start of treatment later in the disease will be able to delay or prevent long-term consequences as renal failure. Vasopressin V2 receptor antagonists are indeed believed to be preventive, not restorative. Furthermore, the dosages of the vasopressin V2 receptor antagonist in the TEMPO ¾ study result in polydipsia, polyuria and nycturia,[64] leading to disturbed night rest and decreased quality of life. Reducing these side effects is important because potentially life long treatment is warranted for ADPKD. Investigating whether lower dosages are associated with fewer side effects, but still significant efficacy in delaying disease

progression will therefore be important.

In an attempt to answer these questions, we administered a vasopressin V2 receptor antagonist to a tamoxifen-inducible kidney epithelium-specific Pkd1-deletion model of ADPKD (chapter 8). This Pkd1-model has very high face validity to the human situation for most patients (85%) have a mutation in the PKD1-gene.[65;66] Of note, the effects of a vasopressin V2 receptor antagonist have never been studied in Pkd1- animal models. We found that indeed, the vasopressin V2 receptor antagonist induced a dose-dependent polyuria in this model. Treatment with this medicament had beneficial effects on cyst growth on short term. When given at similar high dose, prolonged administration of the vasopressin V2 receptor antagonist exhibited less physiologic effect (polyuria), but also less renoprotection. Our results suggest that intervention with a vasopressin-V2 receptor antagonist should be instituted early in the disease course. The fact that diuresis was less increased after prolonged administration, together with the finding in chapter 6, that copeptin concentration is higher in more advanced ADPKD, suggests that it may be necessary to increase the dosage of the vasopressin V2 receptor antagonist when ADPKD progresses.

Future perspectives

We showed, in a cross-sectional study, that copeptin is associated with disease severity in ADPKD. This is in line with the hypothesis that vasopressin induces cyst formation in ADPKD. It would be informative to investigate in a prospective study whether copeptin is also associated with disease progression in ADPKD patients. Disease progression could be assessed in patients who have already advanced disease, as renal function decline. If the study is to be performed in a cohort of ADPKD patients early in the disease, the study should have long-term follow-up (>10 years) to ensure that there are patients under study who experience renal function decline. This question is presently addressed in a cohort of ADPKD patients of whom long-term follow-up is available.[4] Alternatively, disease progression should be monitored by measuring increase in total renal volume, for we confirmed in this thesis that GFR is not an appropriate marker of disease severity in early ADPKD.

Even better than measurement of change in total renal volume is probably measurement of change in renal parenchymal volume. A small-scale study shows that the association between change in parenchymal volume and change in GFR is stronger than between change in total renal volume and change in GFR.[67] Also in our cohort of ADPKD patients, a number of patients have cyst formation mainly on the cortical side of the kidney, growing outward. These patients have a large portion of renal parenchyma preserved and concomitantly (near) normal renal function, despite the large renal volume that is caused by these cortical cysts (see also Figure 2 of chapter 2).

The TEMPO ¾ study (chapter 7) will show whether intervention with a vasopressin V2 receptor antagonist will be able to inhibit or reduce total renal volume growth and improve clinical outcomes in ADPKD patients. If efficacious, we need to learn more about the side effects of this

new drug, especially since therapy has to be given probably life-long for this chronic condition. The results in our animal model suggest that administration of a lower dosage will not only lead to, as intended, fewer side effects (polyuria), but unfortunately also to decreased renoprotection. To overcome this decreased efficacy, combination therapy could be instituted with other agents that are now being tested for ADPKD and that are expected to induce favorable effects by interfering with the same pathophysiological pathways as vasopressin V2 receptor antagonists, such as mTOR inhibitors[68;69] and somatostatin analogues.[11] These latter agents also have side effects that will limit the probability of lifetime use in high dosages. Future research is needed to investigate dose response curves with respect to both renoprotective as well as side effects of all single agents, and of combination therapy.

The TEMPO ¾ study will not answer the question whether intervention with a vasopressin V2 receptor antagonist will also be effective in later phases of ADPKD. A clinical trial will start within short notice in our center, to assess short-term renal hemodynamic effects of vasopressin receptor antagonist in subjects with ADPKD later in the disease (estimated GFR < 60 mL/min). Our results in chapter 8 suggest that early intervention will be more effective than later intervention. After assessment of effect and safety in subjects later in the disease, the question will be who to treat? Should we treat all patients with ADPKD, or only certain subgroups? To prevent exposure to side effects of V2 receptor antagonists in patients with low risk for disease progression, we would encourage, based upon this thesis, to measure effective renal plasma flow, total renal volume, albuminuria and NGAL excretion. Whether these markers will reliably predict disease progression has to be investigated in future prospective research. Furthermore, it would be interesting to investigate whether adequate treatment of ADPKD will induce lowering of NGAL excretion. If this is the case, NGAL excretion could be used as a short-term marker of long-term therapeutic efficacy and even as a marker to titrate dosage of therapeutic strategies.

The finding that patients with more severe ADPKD have higher copeptin levels (chapter 6) indicates that perhaps in these patients higher dosages of vasopressin-V2 receptor antagonist should be administered to effectively suppress endogenous vasopressin levels. In line, we observed in our animal study of ADPKD that after prolonged administration of a selective V2 receptor antagonist, both the physiologic as well as the renoprotective effects of vasopressin inhibitions diminished. What dosage of the V2RA to use could hypothetically be monitored by measuring variables known to be associated with the physiologic effects of vasopressin, as 24h urinary volume, 24h urinary osmolarity and / or fractional urea excretion. Titrating the dosage directed at obtaining a urinary osmolarity less than 300 mOsm/L for instance could be used as a measure of adequate vasopressin inhibition. Whether administration of higher dosages of a vasopressin V2 receptor antagonist to ADPKD patients with lower glomerular filtration will still induce lowering of urinary osmolarity and especially whether this will be safe are important questions that need to be answered.

Apart from the situation in ADPKD, we also showed that copeptin, as surrogate for vasopressin, is associated with albuminuria in healthy subjects and with renal function decline in renal transplant recipients, supporting the general detrimental role of vasopressin in chronic kidney disease that is described in experimental studies.[45;46;70] Whether vasopressin is also associated with renal function decline in other renal diseases is an interesting subject for future studies. In theory, vasopressin might contribute to chronic kidney disease progression by its effect on renal hemodynamics, blood pressure, and mesangial and / or epithelial cells.41 This alludes to the intriguing possibility that interventions to lower vasopressin activity, as administration of vasopressin receptor antagonists or by simply drinking more water, may be beneficial in non-ADPKD chronic kidney disease.

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Samenvatting en toekomst perspectief

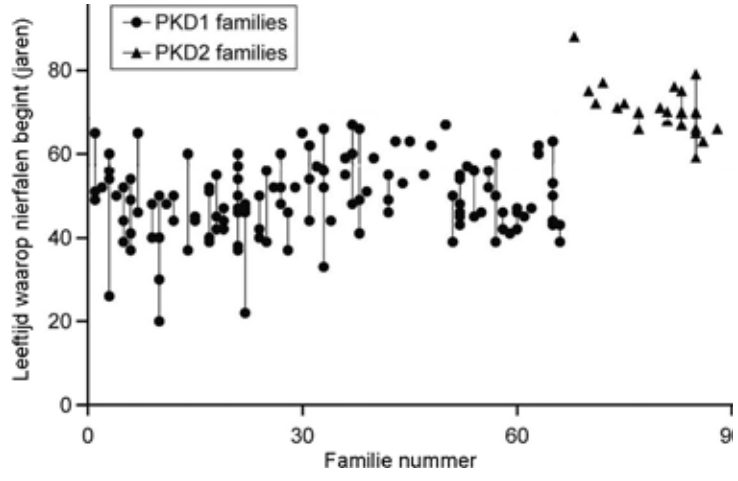
Voor de geïnteresseerde niet-medicus

Samenvatting

ADPKD is de afkorting van Autosomal Dominant Polycystic Kidney Disease. Vrij in het Nederlands vertaald betekent dit niet geslachtschromosoom gebonden, dominant overervende polycysteuze nierziekte, ofwel kortweg cystenieren. ADPKD is de meest voorkomende erfelijke nierziekte. Er zijn 2 genen ontdekt, die als ze beschadigd zijn (een mutatie bevatten), cystenieren kunnen veroorzaken: het PKD-1 en het PKD-2 gen. De ziekte kenmerkt zich door de vorming van cysten (een soort vochtblazen) in een aantal organen, vooral in de nieren (Figuur 1), waardoor de nier na verloop van tijd niet goed meer kan functioneren.

Figuur 1: MRI opname van een man met cystenieren. Het niervolume van deze man is meer dan 3 liter. Voor gezonde mensen ligt het totaal niervolume (dus beide nieren samen) rond de 330 ml. Uit Meijer e.a., NTvG, 2009.

Mensen met cystenieren ontwikkelen over het algemeen tussen hun 40e en 70e jaar ernstig nierfalen, waarvoor nierfunctie vervangende behandeling noodzakelijk is, zoals dialyse of een niertransplantatie. Hierbij moet wel gezegd worden dat de ziekte uiterst variabel verloopt. Sommige patiënten komen op hun 20e levensjaar al in aanmerking voor nierfunctie vervangende behandeling, anderen overlijden op hun 90e levensjaar met een nog redelijk functionerende nier. Figuur 2 laat deze variabiliteit in leeftijd zien waarop nierfalen bereikt wordt. Uit deze afbeelding blijkt dat mensen die uit een familie komen waarin er een mutatie zit in het PKD-2 gen over het algemeen op latere leeftijd nierfalen krijgen dan mensen met een PKD1-mutatie in de familie. Daarnaast valt op dat er verschil is tussen de families, de ene familie komt eerder in dialyse dan de ander. Tot slot, is er ook binnen de families variatie in de leeftijd waarop het nierfalen begint.

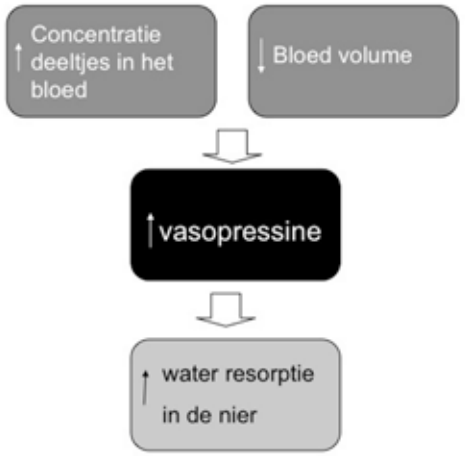


Figuur 2: variatie in leeftijd waarop nierfalen wordt bereikt. Uit Barua e.a., JASN, 2009.

De huidige behandeling bestaat met name uit het voorkomen van complicaties van de ziekte, zoals het geven van bloeddruk verlagende medicatie in het geval van hoge bloeddruk. Op dit moment bestaat er geen behandeling die in staat is om de cystevorming af te remmen en daarmee de nierfunctie te behouden.

De afgelopen jaren zijn er belangrijke ontdekkingen gedaan op het gebied van cystenieren. Zoals gezegd weten we inmiddels in welke genen de mutatie zit die de ziekte veroorzaakt. We weten in welke niet goed functionerende genproducten dit resulteert en hebben aanwijzingen over hoe dit uiteindelijk tot cystevorming leidt. De ontrafeling van deze mechanismen heeft geleid tot mogelijke aangrijpingspunten om cystevorming tegen te gaan of te verminderen. Eén van de mogelijkheden zou het geven van vasopressine V2 receptor antagonisten (afgekort als V2RA) kunnen zijn. Deze medicamenten blokkeren de werking van vasopressine door het blokkeren van de vasopressine V2 receptor. Dit is een receptor die alleen in de nier aanwezig is.

Vasopressine is een lichaamseigen stof die ervoor zorgt dat je lichaam vocht kan vasthouden en niet uitplast. Vasopressine staat ook wel bekend als antidiuretisch hormoon, omdat het dus de diurese (produceren van urine) tegengaat. Zonder vasopressine zou je enorme hoeveelheden water moeten drinken om niet uit te drogen. Figuur 3 geeft de functie van vasopressine in het lichaam schematisch weer. Als de concentratie deeltjes in het bloed hoger wordt, of het volume van bloed afneemt (je droogt een beetje uit), wordt er door je hersenen vasopressine aan de bloedbaan afgegeven (dit gebeurt overigens nog voordat er een dorstprikkel ontstaat). Het vasopressine zorgt ervoor dat er water wordt teruggeresorbeerd in de nier, waardoor het urinevolume omlaag gaat en de concentratie van afvalstoffen in de urine omhoog. Uiteindelijk gaat hierdoor de concentratie van het bloed naar beneden en het bloed volume omhoog, waardoor de afgifte van vasopressine weer geremd wordt. Zo ontstaat er een regelmechanisme waardoor het lichaam zichzelf in evenwicht kan houden wat betreft vochthuishouding.



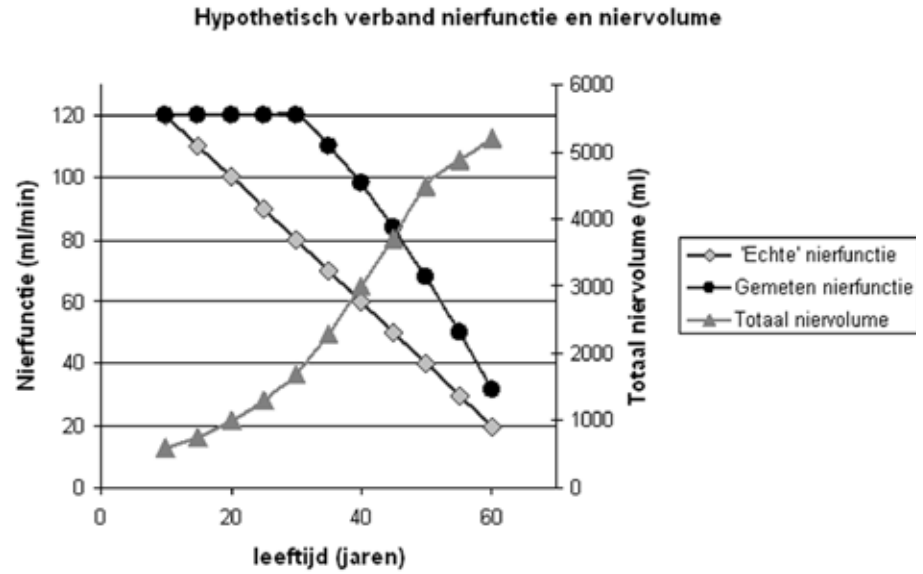
Figuur 3: Schematische voorstelling van de functie van vasopressine

In diermodellen van ADPKD verminderde een V2RA de vorming en groei van niercysten. V2RA's worden al gebruikt bij andere aandoeningen (bij een laag natrium in het bloed en bij hartfalen), waardoor we weten dat ze relatief veilig zijn en wellicht minder bijwerkingen hebben dan veel andere medicamenten waarvan gedacht wordt dat ze mogelijk de vorming van cysten zouden kunnen bestrijden.
Dit proefschrift richt zich op de rol van vasopressine bij het veroorzaken en/of verergeren van cystenieren. Het proefschrift is opgedeeld in drie delen. Het eerste deel beschrijft onze zoektocht naar geschikte variabelen om ziekte-ernst van ADPKD te meten. In het tweede deel wordt onderzocht of vasopressine een schadelijke rol heeft. Hier hebben we naar gekeken bij mensen met cystenieren, maar ook in andere (patiënt)groepen. Het derde deel tenslotte, is een samenvoeging van de twee eerdere delen. Hierin hebben we de effecten van het remmen van vasopressine bij ADPKD bestudeerd.

Deel 1: Het meten van de ziekte-ernst in ADPKD

ADPKD verloopt bij elke patiënt anders. Bij de een houden de nieren op zeer jonge leeftijd op met werken, een ander bereikt dat punt misschien nooit (Figuur 2). Daarnaast hebben alle medicijnen bijwerkingen. Het zou daarom het beste zijn alleen medicijnen te geven aan patiënten bij wie de kans groot is dat ze in korte tijd verergering van hun ziekte krijgen. Het is daarom belangrijk zulke patiënten te kunnen identificeren. Bij veel nierziekten wordt daarvoor de nierfunctie gebruikt. Met nierfunctie wordt dan het klaringsvermogen van de nieren bedoeld, ofwel het vermogen van de nieren om het bloed van afvalstoffen te zuiveren. Dit wordt uitgedrukt als de hoeveelheid bloed die per minuut door de nier van afvalstoffen kan worden gezuiverd. Hiervoor geldt: hoe groter dat bloedvolume, hoe beter de nier werkt. Voor een gezond mens ligt dit rond de 120 ml/minuut. Er zijn echter aanwijzingen dat de nierfunctie in een vroeg stadium van cystenieren een onbetrouwbare maat is voor de ernst van de ziekte. Bij een normaal persoon bestaan de nieren uit 500.000 tot 1 miljoen zogenaamde nefronen. Dit zijn kleinst functionerende eenheden van de nier. Er wordt aangenomen dat de nierfunctie bij patiënten met ADPKD

lang 'normaal' blijft ondanks verlies aan nefronen door cystevorming, doordat de overgebleven nefronen extra hard gaan werken. Er is dus wel functionerend nierweefsel verloren, maar je kunt het niet meten. Figuur 4 illustreert dit fenomeen. Cystevorming, en dus vergroting van de nieren, is een bijna exponentieel verlopend proces (totaal niervolume in Figuur 4) waardoor er nefronen kapot gaan en de nierfunctie afneemt ('echte' nierfunctie). Echter, de de overgebleven nefronen kunnen dit een tijdlang compenseren door harder te werken dan anders, de zogenaamde reserve capaciteit van de nier wordt dan benut. Hierdoor wordt de nierfunctie niet slechter in het begin van de ziekte (gemeten nierfunctie in de figuur), terwijl er wel functionerend nierweefsel verloren is gegaan.



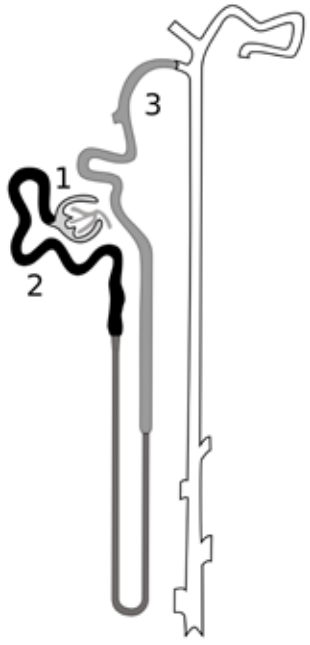
Figuur 4: Hypothetisch verband tussen nierfunctie en niervolume in ADPKD.

Kortom, in ADPKD zijn markers nodig voor ziekte-ernst en die zijn momenteel niet heel duidelijk voorhanden. Deel 1 van dit proefschrift beschrijft onze zoektocht naar markers die geassocieerd zijn met ziekte-ernst.

In hoofdstuk 2 hebben we gekeken naar de nierfunctie en de nierdoorbloeding bij ADPKD patiënten van verschillende leeftijden en hebben die vergeleken met de nierfunctie en nierdoorbloeding van gezonde personen van dezelfde leeftijd. Onze belangrijkste bevinding van dit hoofdstuk is dat al op jonge leeftijd (rond de 28 jaar) de ADPKD patiënten een verminderde nierdoorbloeding hadden in vergelijking met de gezonde personen. Hun nierfunctie was echter wel (bijna) hetzelfde. Dit bevestigt de aanname in de wetenschappelijke literatuur dat nierfunctie in een vroeg stadium van de ziekte geen goede maat is. Ondanks een (bijna) normale nierfunctie, hebben deze jonge cystenierpatiënten namelijk wel degelijk afwijkingen aan hun nieren, zoals een verminderde nierdoorbloeding, vergrote nieren, ze plassen een groter volume urine (wat er waarschijnlijk op duidt dat hun nieren minder goed water kunnen vasthouden en dus urine

minder kunnen concentreren) en er zit meer albumine (een bepaald eiwit wat duidt op nierschade) in hun urine. Tevens geeft dit aan dat in een vroeg stadium van cystenieren beter nierdoorbloeding en niergrootte gemeten kan worden om te bepalen of er daadwerkelijk nierschade is.

Het meten van de nierdoorbloeding gebeurt met radioactieve merker stoffen die worden ingespoten en gemeten in bloed en urine. De niergrootte wordt bepaald met behulp van een MRI scan. Beide metingen nemen veel tijd in beslag, zijn belastend voor de patiënt en zijn duur. Het zou wenselijk zijn een makkelijker te bepalen maat voor de ernst van nierschade te hebben. Door de cystevorming in de nieren ontstaat er een soort ontsteking van diverse onderdelen van de nier. Zoals benoemd bestaat de nier uit vele nefronen. Een nefron bestaat weer uit een soort filter (de glomerulus), waar het bloed gefiltreerd wordt en waar afvalstoffen het lichaam kunnen verlaten, maar ook uit een soort buizensysteem (de tubulus) die aan de glomerulus vast zit, waar de voor-urine doorheenloopt alvorens die in de blaas terechtkomt. In de tubulus vinden allerlei processen van resorptie en uitscheiding plaats, waardoor uiteindelijk de urine wordt gevormd zoals een mens die uitplast. Ook deze tubulus is weer op te delen in een stuk wat dichtbij het filter zit (de proximale tubulus) en een stuk wat juist verder bij het filter vandaan en dichter bij de blaas zit (de distale tubulus). Zie Figuur 5. Globaal bestaat een nefron dus uit 3 delen: de glomerulus (1), de proximale tubulus (2) en de distale tubulus (3).



Figuur 5: Verschillende delen van het nefron. 1: glomerulus, 2: proximale tubulus en 3: distale tubulus. Uit ' klinische nefrologie' van De Jong ea.

Schade aan de verschillende onderdelen van het nefron maakt dat er stoffen worden afgegeven aan de voorurine, wat eventueel te meten is in de uiteindelijke urine. Deze stoffen zouden ideale markers zijn om ziekte-ernst te meten, omdat ze gemakkelijk te verkrijgen zijn uit urine en er dus geen bloed geprikt hoeft te worden. Bovendien is het aannemelijk dat juist in de urine stoffen gemeten kunnen worden die de ernst van een nierziekte weerspiegelen. Urine komt immers rechtstreeks uit de nier. Bij een aantal nierziekten is naar deze stoffen en ziekte-ernst gekeken. Soms is er zelfs gekeken naar deze stoffen en de kans op ziekte-progressie. Ook bij cystenieren is er een aantal van deze stoffen gemeten. Maar altijd maar een per onderzoek, wat vergelijking van de waarde van deze stoffen moeilijk maakt. De metingen zijn bovendien tot nu toe gedaan in betrekkelijk kleine groepen patienten.

Wij hebben daarom bij cystenierpatiënten markers gemeten in de urine die afkomstig zijn uit de glomerulus, uit de proximale- en de distale tubulus en algemene ontstekingsmarkers (hoofdstuk 3). We wilden bekijken of deze markers verhoogd waren ten opzichte van gezonde

mensen. Daarnaast wilden we bepalen of ze een relatie hadden met de ziekte-ernst (uitgedrukt als verminderde nierdoorbloeding en verhoogde niergrootte, omdat we in het vorige hoofdstuk konden bevestigen dat nierfunctie geen goede maat is voor ziekte-ernst. Nierdoorbloeding en niergrootte bleken mogelijk een betere maat te zijn). We vonden dat verschillende markers in de urine verhoogd waren ten opzichte van de gezonde personen. Zowel de markers duidend op algemene ontsteking, als die afkomstig uit de glomerulus, proximale- en distale tubulus waren verhoogd aanwezig in de urine van de ADPKD patiënten. Dit komt waarschijnlijk doordat alle onderdelen van de nier in enige vorm beschadigd zijn. Een aantal markers was geassocieerd met nierdoorbloeding of niergrootte. De marker NGAL, voornamelijk afkomstig uit de proximale tubulus, was als enige geassocieerd met zowel nierdoorbloeding als niergrootte. Cystenierpatienten met een hoog NGAL gehalte in de urine hadden een verminderde nierdoorbloeding en vergrote nieren. Gebaseerd op deze bevinding, zouden we willen aanraden vooral NGAL te meten bij ADPKD patiënten om de ernst van hun nierziekte te beoordelen.

Deel 2: Vasopressine en nierschade

In dit deel hebben we gekeken naar de mogelijk schadelijke rol van vasopressine bij het veroorzaken van nierschade in verschillende (patiënt) groepen. Eerder is al genoemd dat vasopressine heel belangrijk is voor het menselijk lichaam. Zonder vasopressine kan de nier geen water terugresorberen en zou je vele liters urine plassen en bijna continu moeten drinken om niet uit te drogen (Figuur 3). Uit experimentele studies (met cellen en dieren) komt echter naar voren dat vasopressine ook een schadelijk effect zou kunnen hebben op de nieren. Uit voorgaande studies blijkt dat als in ratten met chronisch nierfalen vasopressine wordt geremd (met medicatie of door ze heel veel water te laten drinken), het beter gaat met de nier. Wetenschappelijk onderzoek naar de relatie tussen vasopressine en nierschade bij mensen ontbreekt helaas. Dit zou kunnen komen doordat vasopressine moeilijk te meten is. Vasopressine is gebonden aan bloedplaatjes, is niet stabiel in plasma en veel bepalingen hebben een beperkte gevoeligheid (dat wil zeggen, de tests kunnen beneden een bepaalde grens de hoeveelheid vasopressine niet precies meten). Recent is er een test ontwikkeld welke copeptin meet . Copeptin is een stukje van de voorloper van vasopressine. Het is aangetoond dat de concentratie copeptin gelijk opgaat met de concentratie vasopressine. Copeptin is dus een soort afspiegeling van vasopressine, maar wel eentje die betrouwbaarder te meten is.

Hierdoor waren we in staat te kijken naar de mogelijke relatie tussen vasopressine (indirect gemeten door copeptin te bepalen) en nierschade in verschillende groepen. Eerst hebben we gekeken naar de relatie tussen vasopressine en eiwitverlies in de urine (albuminurie) in de algemene bevolking (hoofdstuk 4). In Groningen wordt het PREVEND onderzoek verricht, waarbij in een grote groep personen in 1997 het eiwitverlies in de urine is gemeten, en waarin nagegaan wordt of dit eiwitverlies een voorspeller is van hart en nierziekten. Mede door dit onderzoek is vast komen te staan dat urine eiwitverlies inderdaad deze aandoeningen voorspelt. Bij 7593 mensen uit dit onderzoek hebben we copeptin gemeten. Bij deze mensen afkomstig uit de bevolking, vonden we ten eerste dat mensen met een hoog vasopressine een laag urinevolume hadden. Dit is ook wat je verwacht, aangezien vasopressine ervoor zorgt dat de nieren water

vasthouden, en je dus minder vocht via de urine verliest (Figuur 3). We vonden verder wat we verwacht hadden, namelijk dat mensen met een hoge vasopressine spiegel ook veel albumine in de urine hadden. Deze relatie was het sterkst aanwezig bij oudere mensen.

In een toevoeging op dit hoofdstuk (hoofdstuk 4.1) beschrijven we dat deze relatie tussen vasopressine en albuminurie onafhankelijk is van zoutinname. Je zou namelijk kunnen denken dat een hoge zoutinname leidt tot een hoge vasopressine spiegel (omdat door een hoge zoutinname de concentratie deeltjes in het bloed toeneemt, zie Figuur 3). Meer zoutinname zou daarnaast ook tot meer nierschade en albuminurie kunnen leiden, maar via een ander mechanisme. Dit verband is in eerder onderzoek aangetoond (overigens ook verricht als onderdeel van de PREVEND studie). De relatie tussen vasopressine en albuminurie zou dan een schijnverband zijn, want eigenlijk is zoutinname de onderliggende factor. In hoofdstuk 4.1 beschrijven we echter dat de eerder gevonden relatie tussen vasopressine en albuminurie onafhankelijk is van zoutinname, wat een schijnverband onwaarschijnlijker maakt.

We hebben ook gekeken of deze gezonde mensen met een hoge vasopressine spiegel ook in de tijd sneller achteruitgaan wat betreft hun nierfunctie dan mensen met een lage vasopressine spiegel. Dit was echter, tegen onze verwachting in, niet het geval. Wellicht dat dit veroorzaakt wordt doordat de gezonde nieren van gezonde mensen niet kwetsbaar genoeg zijn voor eventuele schade die vasopressine zou kunnen veroorzaken. We vonden het verband tussen vasopressine en albuminurie het sterkst in de oudste groep mensen die deelnam aan het PREVEND onderzoek, mensen die wellicht al enige vorm van nierschade hadden. Daarnaast, ook in de dierstudies waarin eerder gekeken was naar de mogelijk schadelijke rol van vasopressine, hadden de muizen of ratten die bestudeerd werden ook altijd enige vorm van nierschade.

Om beter te kunnen kijken naar het mogelijke verband tussen vasopressine en nierfunctie-achteruitgang in de tijd hebben we daarom niertransplantatiepatiënten onderzocht. Mensen die een niertransplantatie hebben ondergaan hebben namelijk altijd enige vorm van nierschade. Deze nierschade kent verschillende oorzaken. Ten eerste is er slechts 1 functionerende nier, die voorafgaand aan de transplantatie een tijdje buiten een menselijk lichaam geweest is, waarbij die minder zuurstof heeft gehad. Ten tweede heeft de medicatie die noodzakelijk is om afstoting te voorkomen ook een nadelig bij-effect op de nier. In het Universitair Medisch Centrum Groningen worden al jaren lang veel niertransplantaties verricht, wat ons de mogelijkheid gaf de relatie tussen vasopressine spiegels en snelheid van nierfunctie-achteruitgang in de loop van de tijd te bestuderen (hoofdstuk 5). We hebben dit kunnen onderzoeken in 548 transplantatiepatiënten. De tijd tussen bloedafname waarin copeptin (als maat voor vasopressine) bepaald is en het moment waarop we uiteindelijk de nierfunctie konden bepalen was ongeveer 6 jaar. We vonden allereerst dat mensen met een hogere concentratie deeltjes in het bloed (een hogere osmolariteit), een hogere vasopressine spiegel hadden. Dit is wat je verwacht te vinden; het hogere vasopressine is bij deze mensen noodzakelijk om water vast te houden, om zo de osmolariteit weer omlaag te brengen (Figuur 3). Evenzo vonden we dat de mensen met een hoog vasopressine een kleiner urinevolume hadden dan mensen met een lage hoeveelheid vasopressine. Ook dit kwam

overeen met onze verwachtingen. Immers, de hoge vasopressine spiegel zet de nieren van deze mensen aan water vast te houden, leidend tot een kleiner urinevolume. Vasopressine regulatie en –actie leek bij deze transplantatiepatiënten dus normaal te functioneren. Onze belangrijkste vraag was of er ook een verband was tussen vasopressine en nierfunctie-achteruitgang in de loop van de tijd. Inderdaad bleek het zo te zijn dat de mensen met de hoogste vasopressine spiegels het snelst achteruit gingen wat betreft hun nierfunctie. Dit zou kunnen betekenen dat een hoog vasopressine inderdaad nierfunctie achteruitgang veroorzaakt.

Eerder is al genoemd dat in diermodellen voor ADPKD een vasopressine V2 receptor antagonist de vorming van cysten verminderde. Oftewel: het remmen van vasopressine heeft een gunstig effect. Logischerwijs is vasopressine dan schadelijk voor de nier bij mensen met ADPKD. Tot nu toe was echter nooit naar gekeken naar vasopressine en ziekte-ernst bij mensen met deze ziekte.

Wij hebben daarom de mogelijke relatie onderzocht tussen vasopressine in het bloed en de ernst van ADPKD. De resultaten van de102 patiënten met ADPKD bij wie we dit konden onderzoeken staan beschreven in hoofdstuk 6. Als maat voor ziekte-ernst hebben we gekeken naar renale bloeddoorstroming, totaal niervolume en uitscheiding van albumine in de urine. We vonden ten eerste dat ook in deze ADPKD patiënten de concentratie van de hoeveelheid deeltjes in het bloed geassocieerd was met vasopressine concentratie. In tegenstelling tot de situatie bij gezonde mensen of transplantatiepatiënten, vonden we echter geen duidelijk verband tussen vasopressine en de hoeveelheid en / of de concentratie van de urine. Dit zou een aanwijzing kunnen zijn dat vasopressine niet zijn normale fysiologische effecten heeft in deze patiënten. Het antwoord op onze belangrijkste onderzoeksvraag is dat er inderdaad een duidelijk verband bleek tussen vasopressine en ziekte-ernst: hoe hoger het vasopressine, hoe lager de bloeddoorstroming van de nieren, hoe groter de nieren en hoe meer albumine uitscheiding in de urine. Deze verbanden golden voor zowel mannen als vrouwen, van alle leeftijden.

Deel 3: Kunnen vasopressine V2 receptor antagonisten nierschade bij ADPKD remmen?

Dat er een verband bestaat tussen vasopressine en ziekte-ernst in ADPKD past goed bij de eerder genoemde resultaten in diermodellen van deze ziekte. Hierin gaf het remmen van vasopressine met behulp van zogenaamde Vasopressine V2 receptor antagonisten (V2RA) een gunstig resultaat. Dit biedt wellicht ook een goed vooruitzicht voor een grote klinische studie die op dit moment uitgevoerd wordt. Deze studie heet de TEMPO ¾ studie. TEMPO is een afkorting die staat voor Tolvaptan Efficacy and Safety in Management of Autosomal Dominant Polycystic Kidney Disease and its Outcomes, phase 3 of 4. De studie onderzoekt of een V2RA (Tolvaptan, merknaam Samsca®) een gunstig effect kan hebben op de niergroei bij patiënten met ADPKD. In hoofdstuk 7 wordt de studie-opzet en de achterliggende gedachten bij deze opzet besproken. Aan deze studie mogen ADPKD patiënten meedoen die jong zijn, nog een goede nierfunctie hebben, maar al wel een groot niervolume. De reden hiervoor is dat verwacht wordt dat juist deze mensen een ernstiger beloop van hun nierziekte zullen hebben, waardoor het mogelijk zal zijn een eventueel gunstig effect van de studiemedicatie te vinden. Eén van de drie mensen die

deelneemt aan de studie krijgt tolvaptan, de ander krijgt placebo (een tablet die er hetzelfde uitziet, maar geen werkzame stof bevat). Het slikken en geven van de medicatie gaat dubbel blind. Dat wil zeggen dat de deelnemer niet weet of hij/zij placebo of tolvaptan slikt, maar ook de onderzoeksdokters niet weten wat ze aan de patiënt geven. De studie zal drie jaar duren. Belangrijkste uitkomstmaat van de studie is het verschil in verandering van niervolume bij patiënten die tolvaptan slikken versus patiënten die placebo slikken gedurende de 3 jaar van deelname aan het onderzoek. Daarnaast wordt ook gekeken naar verschil tussen deze twee groepen in verandering van nierfunctie, toename van albumine uitscheiding in de urine en nierpijn. Tussen maart 2007 en januari 2009 zijn er wereldwijd 1445 ADPKD patiënten aan deze studie begonnen, 746 mannen en 699 vrouwen. In het Universitair Medisch Centrum Groningen, zijn er 52 patiënten die deelnemen aan de studie. Ons centrum is daarmee het op 1 na grootste centrum. Tot nu toe hebben we alleen beschikking over de resultaten aan het begin van de studie. Inderdaad hebben deze 1445 mensen nog een goede nierfunctie. De klaring van hun nieren wordt geschat op 105 mL/min, wat een bijna normale waarde is. Hierbij moet wel gezegd worden dat de spreiding in nierfunctie groot is, er zijn dus ook patiënten bij die een beduidend lagere nierfunctie hebben. Het niervolume is groot (mediaan 1,5 liter, ongeveer 5 keer zo groot als het niervolume van gezonde mensen) en de leeftijd laag (gemiddeld ongeveer 39 jaar). Omdat er zoveel patiënten deelnemen aan dit onderzoek en ze inderdaad jong zijn, een goede nierfunctie hebben, maar een groot niervolume, is de kans groot dat deze studie een antwoord zal kunnen geven op de vraag of vasopressine V2 receptor antagonisten een gunstig effect hebben op de nieren van ADPKD patiënten.

Als uit deze studie blijkt dat vasopressine V2 receptor antagonisten effectief zijn bij ADPKD, is er echter nog een aantal belangrijke vragen te beantwoorden. Aan de TEMPO studie mogen alleen ADPKD patiënten deelnemen als ze in een vroeg stadium van de ziekte zijn. De patiënten moeten namelijk jong zijn en nog een relatief goede nierfunctie hebben. Het blijft de vraag of dit medicament ook effectief is als er later in de ziekte mee begonnen wordt. Daarnaast geeft een V2RA toename van de wateruitscheiding, dus mensen moeten meer en vooral vaker plassen. Ook 's nachts moeten ze daarvoor (vaker) hun bed uit. Dit is niet in elk beroep even makkelijk en zou daarnaast kunnen leiden tot een minder goede nachtrust en daarmee een vermindering van de kwaliteit van leven. Omdat behandeling met dit medicament waarschijnlijk gedurende het hele leven zal moeten plaatsvinden, is het belangrijk dat de bijwerkingen niet al te hinderlijk zijn. Het is daarom van belang te onderzoeken of lagere dosering van het medicament tot minder bijwerkingen zal leiden, maar daarnaast nog wel effectief is wat betreft gunstig beïnvloeden van het beloop van hun nieraandoening.

In een poging de vragen te beantwoorden over dosering en moment van toedienen, hebben we een vasopressine V2 receptor antagonist aan muizen gegeven die ADPKD hebben. Dit wordt beschreven in hoofdstuk 8. De ziekte in de muizen die wij gebruikt hebben, lijkt sterk op die bij mensen en wordt ook veroorzaakt door hetzelfde genetische defect (een mutatie van het PKD1-gen). We vonden dat behandeling met een V2RA inderdaad resulteerde in een vermindering van de cystegroei en een vermindering van het niervolume bij deze muizen op korte termijn (d.w.z. 3 weken behandelen). Het op een later tijdstip geven van het medicament, of in een lagere dosering, resulteerde wel in minder bijwerkingen (minder urine volume), maar helaas ook in minder bescherming tegen cystvorming. Als de behandeling die vroeg werd begonnen, in hoge dosering, voortgezet werd, werd het eerder gevonden gunstige effect op de nier minder duidelijk. Ook hadden de muizen later minder bijwerkingen van het medicament dan ze aanvankelijk hadden (zo dronken ze minder, gingen ze minder plassen en werd de urine weer geconcentreerder). Dit zou erop kunnen wijzen dat vasopressine niet helemaal onderdrukt is in deze muizen. Wellicht is het noodzakelijk in het vervolg een hogere dosering te geven van dit medicijn naarmate de ziekte vordert.

Toekomstperspectief

In dit proefschrift hebben we aangetoond dat copeptin geassocieerd is met ernst van ADPKD (hoe hoger de vasopressine concentratie, hoe ernstiger de ziekte). Dit past bij de theorie dat vasopressine ook daadwerkelijk groei van cysten in ADPKD bevordert. Het zou heel interessant zijn te kijken of ADPKD patiënten die nu een hoog vasopressine hebben, er niet alleen op dit moment slechter aan toe zijn, maar ook sneller achteruitgaan in de loop van de tijd. Het is natuurlijk moeilijk hoe je dan achteruitgang in de tijd moet uitdrukken, zeker nu we weten dat nierfunctie lang 'normaal' blijft in deze ziekte, terwijl de nieren van deze patiënten in werkelijkheid wel achteruitgaan (Figuur 4). Achteruitgang zou daarom gemeten kunnen worden als nierfunctie-achteruitgang in een groep patiënten die al een gestoorde nierfunctie hebben. Ook zou dit onderzocht kunnen worden bij patiënten die in een eerder stadium van de ziekte zitten, maar dan door het niervolume te meten of door de mensen heel lang in de tijd te vervolgen. Op dit moment zijn we aan het kijken of we deze vraag (of er een verband is tussen vasopressine en nierfunctie-achteruitgang in ADPKD patiënten) kunnen beantwoorden met behulp van een groep ADPKD patiënten die ongeveer 15 jaar geleden aan een studie in het Universitair medisch Centrum Leiden hebben meegedaan.

Hopelijk zal de TEMPO ¾ studie gaan aantonen dat een vasopressine V2 receptor antagonist (V2RA) effectief is bij ADPKD wat betreft het remmen van groei van totaal niervolume. We zullen dan goed moeten kijken naar de bijwerkingen van dit medicament. Te meer daar het waarschijnlijk levenslang gegeven zal moeten worden. De resultaten in het diermodel (hoofdstuk 8) suggereren dat een lagere dosis misschien wel leidt tot minder bijwerkingen, maar helaas ook tot een minder gunstig effect op de nier. Om nog echt effectief te zijn, zou er gekeken moeten worden naar de mogelijkheid een vasopressine V2 receptor antagonist te combineren met andere medicamenten waarvan nu de effectiviteit bij cystenieren getest wordt. Te denken valt dan met name aan zogenaamde somatostatine-analogen. Belangrijk is te vermelden dat ook deze medicamenten bijwerkingen hebben. In toekomstig onderzoek zal er gekeken moeten gaan worden naar optimale medicatiedosis, zodat er wel effect is, maar zo min mogelijk bijwerkingen. Het lijkt verstandig dit te onderzoeken van alle medicamenten apart, maar zeker ook van combinatietherapie.

Een andere vraag die door de TEMPO ¾ studie niet beantwoord gaat worden is of de vasopressine V2 receptor antagonist ook nog effectief is in een later stadium van de ziekte. De resultaten uit hoofdstuk 8 suggereren dat eerdere interventie effectiever lijkt dan later. Binnenkort zal er in ons centrum een onderzoek starten waarin we willen kijken naar veiligheid en effectiviteit van dit medicament bij mensen met een slechte nierfunctie.

De volgende vraag is dan: wie moeten we behandelen? Aangezien ADPKD een erfelijke ziekte is, weten we soms al als kinderen heel jong zijn of ze ADPKD hebben. Moet dan direct behandeling gestart worden? Omdat de ziekte erg variabel is en om te voorkomen dat iedereen wordt blootgesteld aan bijwerkingen van medicijnen, zou het het mooist zijn als we zouden kunnen voorspellen wie vroeg in zijn leven moet gaan dialyseren of in aanmerking komt voor een transplantatienier. Met andere woorden, wie het meeste baat heeft bij behandeling met een dergelijk medicament. Helaas kunnen we dat op dit moment nog niet. Wel kunnen we aanraden, op grond van onze bevindingen in hoofdstuk 3, te onderzoeken of NGAL een marker is die ziekte progressie wellicht kan voorspellen. Het zou ook interessant zijn te kijken of NGAL omlaag gaat op het moment dat behandeling aanslaat.
In dat geval zou NGAL gebruikt kunnen worden als marker om op korte termijn te kunnen beoordelen wat het effect van medicatie is op de lange termijn.

Onze bevinding dat mensen met ernstiger ADPKD hogere vasopressine spiegels hebben dan mensen met minder ernstig ADPKD (hoofdstuk 6), geeft aan dat deze mensen misschien hogere doses van een vasopressine V2 receptor antagonist zouden moeten krijgen om hun vasopressine spiegels volledig te onderdrukken. In de dierstudie (hoofdstuk 8) bleek ook dat het geven van een V2RA aan deze muizen gedurende langere tijd op den duur geen gunstig effect meer had, maar ook minder bijwerkingen gaf. Wellicht dat het ophogen van de dosis een oplossing zou zijn. Welke dosis dan het beste gegeven kan worden zou wellicht bepaald kunnen worden op basis van de concentratie van de urine (als de urine te geconcentreerd is, moet de dosering omhoog). Of dit altijd veilig is en echt werkt, zal in toekomstige studies uitgezocht moeten worden.
Naast de situatie in ADPKD, toonden we ook aan dat er een verband bestaat tussen hoge vasopressine spiegels en albumine uitscheiding in de urine in gezonde personen. Bij mensen met een transplantatienier vonden we een verband tussen vasopressine en nierfunctie-achteruitgang (waarbij de mensen met de hoogste vasopressine spiegels de meeste albumine uitscheiding en de meeste nierfunctie-achteruitgang hadden). Ook in eerder verrichtte dierstudies zijn er aanwijzingen dat een hoog vasopressine slecht zou kunnen zijn voor de nier. Vasopressine zou mogelijk kunnen leiden tot een hoge bloeddruk en een hoge druk in de nier. Dit draagt bij aan de intrigerende gedachte dat acties die de vasopressine concentratie in het bloed remmen (door een V2RA te slikken, maar ook simpelweg door meer water te drinken) een gunstig effect zouden kunnen hebben op de nier bij mensen met een chronische nierziekte van andere oorsprong dan ADPKD.

Kortom, uit deze laatste paragraven blijkt weer eens dat onderzoek weer nieuw onderzoek oproept. Maar ook wordt duidelijk uit deze samenvatting dat de hoop groeit dat er eindelijk een behandeling komt die nierfunctie achteruitgang kan vertragen bij patiënten met ADPKD.

Dankwoord

Dankwoord

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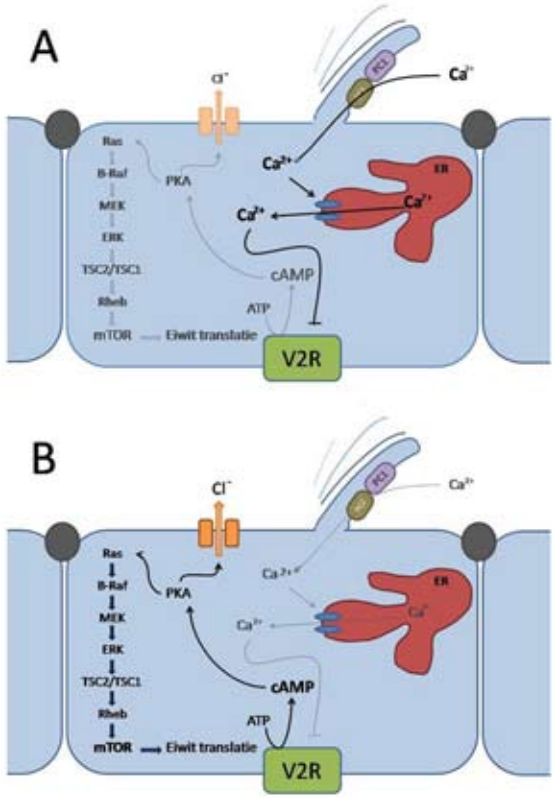
Esther, 2011.

Chapter 1: Figure 7: Hypothetical pathogenetic pathways in ADPKD.



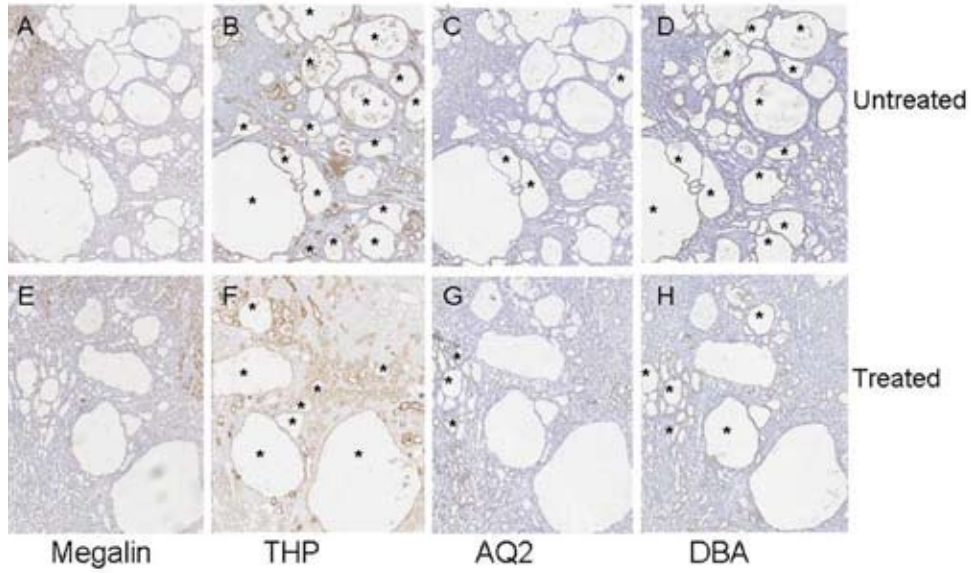
Pathways depicted in bold are upregulated, pathways depicted in grey are downregulated.

A. Depicts an epithelial cell of the kidney's collecting duct of a healthy individual. As long as there is urine flow in the tubule, the cilium will bend, inducing a calcium influx through the polycystin complex. This in turn induces release of calcium from the endo plasmatic reticulum. Cyclic AMP is inhibited by this calcium.

B. Depicts this same cell, but now derived from an individual with ADPKD. Because of a defect in the polycystin complex, there is less calciuminflux, resulting in an increased intracellular cyclic AMP concentration. This will induce on the one hand more protein translation, causing a disturbed proliferation, adhesion, migration, differentiation and maturation of the tubulule cells and on the other hand more chloride dependent fluid production, enabling the cysts to grow.

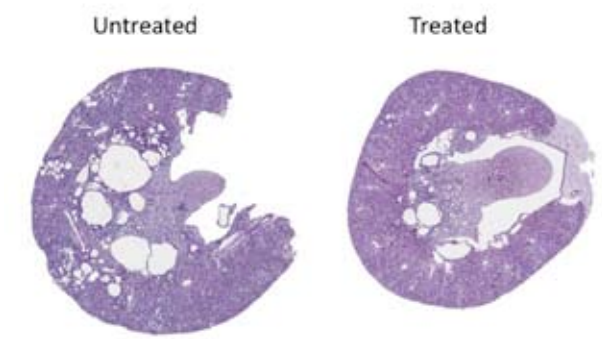
How these processes are interrelated is at this moment still unknown. Vasopressin V2 receptor antagonists block the vasopressin V2 receptor (V2R) and thereby inhibit cAMP, inhibitors of mTOR inhibit protein translation.

Chapter 8: Figure 4: Tubular segment identity of cysts.



Panels A-D show kidney sections from an untreated iKsp-Pkd1del mouse, sacrificed at 3 weeks. Panels E-H show kidney sections from a mouse treated with a high dose V2RA for 3 weeks. Sections are stained for the proximal marker Megalin (A&E), the distal markers Tamm Horsfall Protein (THP, B&F), Aquaporin-2 (AQ2, C&G), and lectin Dolichos biflorus agglutinin (DBA, D&H). * indicates positive staining of the cyst.

Chapter 8: Figure 5: Renoprotective effects of the vasopressin V2 receptor antagonist (V2RA) at week 3 and week 6 of the experiment.



Cyst ratio (upper panel) and kidney weight (middle panel) for experimental groups. Data are presented as means and standard errors of the mean. No statistically significant differences between the various ADPKD groups at week 6 were found. * indicates $p<0.05$ compared with wild-type. HD, high dose (0.1%).
The lower panel shows representative kidney sections of an untreated iKsp-Pkd1del mouse and iKsp-Pkd1del mouse treated with high dose V2RA at week 3 of the experiment.